Mosquito-borne Arboviruses of Horses:

Vector Presence, Competence and Disease Prevention in the UK.

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy

by

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This thesis is based on research carried out in the Department of Epidemiology and Population Health at the University of Liverpool. Except where indicated, the content of this thesis is my own work.

Gail Chapman

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"Think like a (wo)man of action. Act like a (wo)man of thought." – Henry Bergson

Abstract

Mosquito-borne arboviruses cause significant morbidity and mortality in horses worldwide and can have substantial welfare and economic ramifications. Eight main arboviruses of equids are discussed in this thesis: the flaviviruses West Nile Virus (WNV), Japanese encephalitis virus (JEV), Murray Valley encephalitis virus (MVEV), and the alphaviruses Eastern equine encephalitis, Western equine encephalitis virus (WEEV), Venezuelan equine encephalitis virus (VEEV), Ross River virus (RRV), and Getah virus (GETV). Except for Getah virus, these viruses cause disease in humans as well as equids. To investigate the risk to horses in the UK, work included in this thesis comprised investigation of mosquito species presence on equine premises, and assessment of vector competence for equine arboviruses. Strategies for protection of horses from mosquito-biting were investigated, and the knowledge of horse owners with regards to equine arboviral diseases, vectors and control and prevention was explored, as this knowledge is important in disease preparedness, surveillance and control.

Known and potential vectors of equine arboviruses are present on equine premises across England. The most widespread was found to be *Culiseta annulata*, which was also demonstrated to be a competent laboratory vector for JEV and has been shown previously to be competent for WNV. The most abundant species trapped was *Ochlerotatus detritus*, which has been previously shown to be a competent vector for some flaviviruses (JEV, WNV) and was demonstrated here to be laboratory competent for RRV. Container habitats of *Culex pipiens* were commonly found on equine premises and this species was shown here to transmit JEV at high rates at 18 °C, which represents average temperatures which may be experienced in a warm summer period in the south of England. Both *Cs. annulata* and *Oc. detritus* were only inefficient laboratory vectors of epizootic VEEV. Apparent virus clearance and nonlinear temperature-transmission relationships were demonstrated for several virus-vector pairs studied.

Horse-owner knowledge of equine arboviral disease was shown to be limited. Spray repellents were shown to have some benefit in the protection of individual horses from mosquito biting, and the potential for some degree of utility in reducing the risk of infection by arboviruses, under low to moderate infection pressure, and in situations in which there is no vaccine available.

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List of Abbreviations

Ae.	*Aedes*
AHS	African horse sickness
AHSV	African horse sickness virus
BUNV	Bunyamwera virus
An.	*Anopheles*
C.	*Culicoides*
°C	degrees Celcius (Centigrade)
CI	confidence interval
CDC	Centers for Disease control and Prevention (USA)
cDNA	complementary deoxyribonucleic acid
COI	cytochtome c oxidase subunit 1
Cq.	*Coquilletidia*
Cs.	*Culiseta*
CT value	threshold cycle value
Cx.	*Culex*
Cx. pipiens	*Culex pipiens pipiens*
DEET	N,N-Diethyl-meta-toluamide
DNA	deoxyribonucleic acid
EEE	Eastern equine encephalitis
EEEV	Eastern equine encephalitis virus
EEV	Equine encephalosis virus
EIP	extrinsic incubation period
GETV	Getah virus
GLM	Generalised Linear Model(ling)
HJV	Highlands J virus

JEV	Japanese encephalitis virus
LIV	Louping ill virus
m	metre
µl	microlitre
ml	millilitre
n	sample size
MVE	Murray Valley encephalitis
MVEV	Murray Valley encephalitis virus
Oc.	*Ochlerotatus*
OIE	World Organisation for Animal Health
P	(P-value) probability value
PCR	polymerase chain reaction
POWV	Powassan virus
PMD	p-Menthane-3,8-diol
rcf	relative centrifugal force
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RRV	Ross River virus
SD	standard deviation
SHUV	Shuni virus
s.l.	sensu lato
SLEV	Saint Louis encephalitis virus
spp.	species (plural)
SSHV	Snowshoe Hare virus
UK	United Kingdom
USA	United States of America
VEE	Venezuelan equine encephalitis
VEEV	Venezuelan equine encephalitis virus

WEE	Western equine encephalitis
WEEV	Western equine encephalitis virus
WNE	West Nile encephalitis
WNV	West Nile virus
x g	times gravity

1 GENERAL INTRODUCTION

Introduction

The rate of emergence of infectious diseases, in particular vector-borne viral diseases such as dengue, chikungunya, Zika, Rift Valley fever, West Nile, Schmallenberg and bluetongue, is increasing globally in human and animal species for a variety of reasons. These include increased movement of animals and people worldwide, environmental and climate change, and human encroachment into natural habitats (taking domestic species with them). Equine arboviruses are no exception to this trend, and a number of authors have highlighted the potential for the introduction of various equine arboviruses to Europe (Durand et al., 2013; Pages et al., 2009; Pfeffer and Dobler, 2002).

Arboviruses are defined as viruses transmitted by biting arthropods, which include mosquitoes, biting midges (*Culicoides*) and ticks. Arboviruses replicate in the body of the insect and are, therefore, distinct from viruses that are transmitted between hosts on or in the mouthparts of insects without replication (termed mechanical transmission), such as equine infectious anaemia virus. The equine arboviruses discussed in this thesis are listed, with their abbreviations, in Table 1.1. Although there are similarities in the transmission cycles of some of these viruses, the details are virus-specific and some are particularly complex. The majority of mosquito-borne equine arboviruses are zoonotic.

Equine Arboviruses

1.2.1 Alphavirus and Flavivirus Disease

The most commonly detected flavi- and alpha-viruses that cause clinical disease in horses worldwide share similarities: they are transmitted by mosquitoes, and viruses of both genera also cause encephalitic disease in both horses and humans. Major flaviviruses known to cause clinical disease in equines include Japanese encephalitis virus (JEV), West Nile virus (WNV) and Murray Valley encephalitis virus (MVEV). Major alphaviruses of horses include eastern, western and Venezuelan equine encephalitis (EEEV, WEEV and VEEV, respectively), Ross River virus (RRV) and Getah virus (GETV) (Table 1.1).

Family/genus	Virus	Abbr.	Important vectors	Zoonotic	Major hosts involved in transmission
Toga~/Alpha	Eastern equine encephalitis	EEEV	*Culiseta melanura*, *Aedes taeniorhynchus* [1]	Y	Passerine birds, rodents
	Getah	GETV	*Aedes vexans niponii*, *Culex* spp. [2;3]	N	Swine
	Ross River	RRV	*Aedes camptorhyncus*, *Culex annulirostris* [4]	Y	Marsupials
	Western equine encephalitis	WEEV	Culex tarsalis, Culiseta melanura [1]	Y	Passerines
	Venezuelan equine encephalitis	VEEV	Enzootic form, *Culex melanoconion* spp. Epizootic form – wide vector range including *Psorophora* and *Ochlerotatus* spp. [5]	Y	Rodents
	Highlands J		*Culiesta melanura* [6]	Y	Passerine birds
Flavi~/Flavi	Japanese encephalitis	JEV	*Culex tritaeniorhyncus*, *Culex vishnu* complex spp. *Culex gelidus* [7;8]	Y	Waterbirds
	Murray valley encephalitis	MVEV	*Culex annulirostris* [9]	Y	Waterbirds
	West Nile virus	WNV	Many *Culex* spp. some of the most important include *Culex pipiens*, *Cx. tarsalis*, *Culex modestus*, *Culex quinquefasciatus* [10;11;12]		Birds[a]
	Powassan	POWV		(Y)	Small mammals
Bunya~/Orthobunya~	Shuni	SHUV	*Culex theilieri?* *Culicoides* spp. [13]	(Y)	Small mammals
	Snowshoe hare	SSHV	*Aedes* spp., *Culiseta* spp. [14;15]		
	Bunyamwera	BUNV	*Aedes* spp. [16]	N	Birds?

Table 1.1 Equine arboviruses described in this thesis.

The eight major viruses of horses upon which this thesis focusses are underlined.

[a] WNV has a broad host range, but ecological significance of rodent and reptile hosts may vary according to region.

1 - (The Walter Reed Biosystematics Unit, 2014); 2 - (Fukunaga et al., 2000); 3 - (Kumanomido et al., 1982); 4 - (Russell, 2002); 5 - (Weaver et al., 2004); 6 - (Borland et al., 2016); 7 - (Gresser et al., 1958); 8 - (Impoinvil et al., 2013); 9 - (Boyle et al., 1983); 10 - (Petersen, 2015); 11 - (Hubálek and Halouzka, 1999); 12 - (Medlock et al., 2012b); 13 - (van Eeden et al., 2012); 14 - (Beaty and Bishop, 1988); 15 - (Newhouse et al., 1971); 16 – (Tauro et al., 2009).

Clinical signs in horses that are infected with the encephalitic viruses (WNV, JEV, MVEV, EEEV, WEEV, and VEEV) include a variety of neurological abnormalities. There is a significant degree of overlap between clinical presentation of these diseases (clinical signs of ataxia and paresis are common to all of them), which can present a challenge in terms of diagnosis (Table 1.2).

Clinical sign	Virus							
	WNV[1]	JEV[2]	MVEV[3]	EEEV[4]	WEEV[4]	VEEV[5]	RRV[6]	Getah[7]
Pyrexia	✓	✓	✓	✓	✓	✓	✓	✓
Gait abnormalities / ataxia	✓	✓	✓	✓	✓	✓	✓	
Paresis	✓	✓	✓	✓	✓	✓		
Paralysis	✓	✓	✓	✓		✓		
Hyperexcitability		✓		✓	✓	✓		
Impaired vision / blindness	✓	✓		✓		✓		
Seizures	✓			✓		✓		
Dromomania / circling		✓	✓					
Muscle fasciculations	✓	✓	✓					
Inability to swallow		✓				✓		
Head pressing				✓		✓		
Colic-like signs	✓		✓					
Stiffness							✓	✓
Oedema							✓	✓
Lymphadenopathy							✓	✓
Flaccid lips / tongue protrusion		✓		✓				
Urticaria / skin eruptions								✓
Hypersensitivity	✓							
Cataplexy / narcolepsy	✓							
Cranial nerve deficits	✓							
Neck rigidity		✓						
Radial paralysis		✓						
Profuse sweating		✓						
Teeth grinding				✓				
Low head carriage				✓				
Drooping ears				✓				
Swollen eyelids				✓				

Table 1.2 Common and important clinical signs of mosquito-borne arboviral diseases in horses.

1 - (Long, 2007); 2 - (Burns and Matumoto, 1949; Ellis et al., 2000; Long, 2007; Onmaz et al., 2013); 3 - (Holmes et al., 2012); 4 - (Long and Gibbs, 2007); 5 - (Henderson et al., 1971; Long and Gibbs, 2007; Taylor and Paessler, 2013); 6 - (El-Hage et al., 2008); 7 - (Fukunaga et al., 2000).

Equine morbidity and mortality information for mosquito-borne viruses affecting horses is presented in Table 1.3.

	Virus							
	JEV	**WNV**	**EEEV**	**WEEV**	**VEEV**	**MVEV**	**RRV**	**Getah virus[1]**
Inapparent infections common	Yes[2]	Yes[3]	Yes[4]	Yes	No[2]	Yes[5]	Yes[6]	Yes
Morbidity	0.03-1.4% of horses in a region[7]	1 in 11-12 infections[3]	61% of horses on some farms[8]	Low	10% of regional population (estimated)[9,10]	Low	Low	Unknown
Case Mortality	5-40%[11,12,13]	38-57%[3]	Up to 73%[8]	20-30%[14]	40-90%[9,10]	Low	Low	Not fatal
Vaccination Available	Yes	UK licensed	Yes	Yes	Yes			Yes

Table 1.3 Mosquito-borne viruses affecting horses and known morbidity and mortality information.

1. (Fukunaga et al., 2000)
2. (Rico-Hesse, 2000)
3. (Delcambre and Long, 2014)
4. (Pauvolid-Corrêa et al., 2010)
5. (Holmes et al., 2012)
6. (Vale et al., 1991)
7. (Spickler, 2010)
8. (Silva et al., 2011)
9. (Sudia et al., 1975a)
10. (Zehmer et al., 1974)
11. (Ellis et al., 2000)
12. (Hale and Witherington, 1953)
13. (Nakamura, 1972)
14. (Long and Gibbs, 2007)

Inapparent infections with limited clinical signs (e.g. transient pyrexia) after infection, are common with the encephalitic viruses and may not be picked up by the owner as illness, or only transient low-grade illness or poor performance. These horses are not, therefore, presented for diagnosis to a veterinarian and will not contribute to case numbers. Therefore, the proportion of deaths per diagnosed cases (case-fatality rate) can be very high. For example, the average fatality rate reported for cases of West Nile encephalitis in the United States between 1999 and 2006 was 30–40% (Delcambre and Long, 2014). However, retrospective estimates suggest that less than 10% of infected horses develop encephalitis. This figure was confirmed in a prospective study involving 37 unvaccinated horses in which only 2 of 25 animals (8%) that seroconverted developed encephalopathy (Gardner et al., 2007). Although around 80% of surviving horses recover from West Nile encephalitis in 3–4 weeks, a small proportion have residual neurological deficits (Ward et al., 2005). In contrast to WNV, JEV, EEV and VEEV, morbidity rates in horses infected with MVEV and RRV (Delcambre and Long, 2014; Long and Gibbs, 2007) are low and they rarely cause fatal disease. Getah virus infection is often subclinical with clinically affected horses usually recovering completely (Fukunaga et al., 2000).

1.2.2 General Mosquito Life Cycle and Role in Transmission

Vectorial capacity is a measure of the efficiency of vector-borne disease transmission by a vector population. Aspects of vectorial capacity in relation to environmental temperature are discussed. This is relevant with respect to the UK as consideration of climate change and the potential for related increases in mosquito populations have led to increased interest in the risk of arboviral disease (Medlock et al., 2005). Relevant aspects of individual British candidate vector species life cycles are discussed later in this chapter.

Vectorial capacity is usually expressed as the number of infective bites received daily by a single host. It is comprised of the average daily vector biting rate (the daily probability of a vector feeding on a susceptible host), vector competence (the proportion of vectors capable of being infected; this may be determined genetically and environmentally), the extrinsic incubation period, and vector lifespan, all of which depend on temperature to a greater or lesser extent.

Adult female mosquitoes feed on blood to enable egg production. They become infected when they feed on a viraemic host and, following virus replication in the vector and spread to the salivary glands, transmitting the virus when they subsequently feed on a susceptible host. Therefore, the vector biting rate depends on the time between laying egg batches (the gonotrophic cycle length). As the ambient temperature increases, the gonotrophic cycle decreases, leading to more frequent feeding, and creating more opportunities for onward transmission of virus. Increases in temperature generally accelerate mosquito development (Rueda et al., 1990), thereby reducing longevity. The impact of raising temperature is greater on some species than others, but the reduction in longevity has been suggested to reduce the overall effects of temperature in increasing vectorial capacity (Ciota et al., 2014).

The extrinsic incubation period (EIP) is defined as the time between a vector obtaining an infective blood meal and being able to infect a susceptible host. The EIP for arboviruses has been shown to be temperature dependent, but it is not a straightforward relationship; EIP shortens with increasing environmental temperature up to a maximum transmission efficiency, though some studies detect a subsequent decrease as the temperature rises further (Hurlbut, 1973; Xiao et al., 2014). There may also be strain differences in the relationship between temperature and EIP. For example, WNV is thought to have expanded rapidly across the US because of genome changes in the strain that became established resulting in a shorter EIP in *Culex* mosquitoes than the strain that was originally introduced (Ebel et al., 2004; Moudy et al., 2007).

The vector biting rate also depends on the density of vectors in relation to the density of the host. Increases in host-vector interaction may result from other consequences of global change, for example decreased rainfall has been associated with a higher incidence of WNV infection in horses (Crowder et al., 2013) and wetland expansion schemes could increase host exposure to mosquitoes if not well managed (Medlock and Vaux, 2011).

1.2.3 Flavivirus epidemiology and ecology

The flaviviruses that cause clinical disease in horses share characteristics in their transmission cycles. In general, these viruses are maintained in an enzootic cycle (i.e. they are transmitted between wild animals, usually birds) and horses (and humans) are infected as "incidental" or "dead-end" hosts (Figure 1.1). Dead-end host species do not (or individuals rarely) produce sufficiently high viraemia to infect mosquitoes, and therefore these species are not considered to be involved in significant ongoing transmission. However, in some situations it may be possible for these species to be involved in the mosquito infections: it has been demonstrated in the laboratory that it is possible for infected mosquitoes to infect naïve mosquitoes with WNV through simultaneous feeding on a host (without viraemia) (Higgs et al., 2005). Although the reservoir hosts are avian, large outbreaks of JEV may be associated with efficient amplification of virus in pigs, which also produce high levels of viraemia (Scherer et al., 1959).

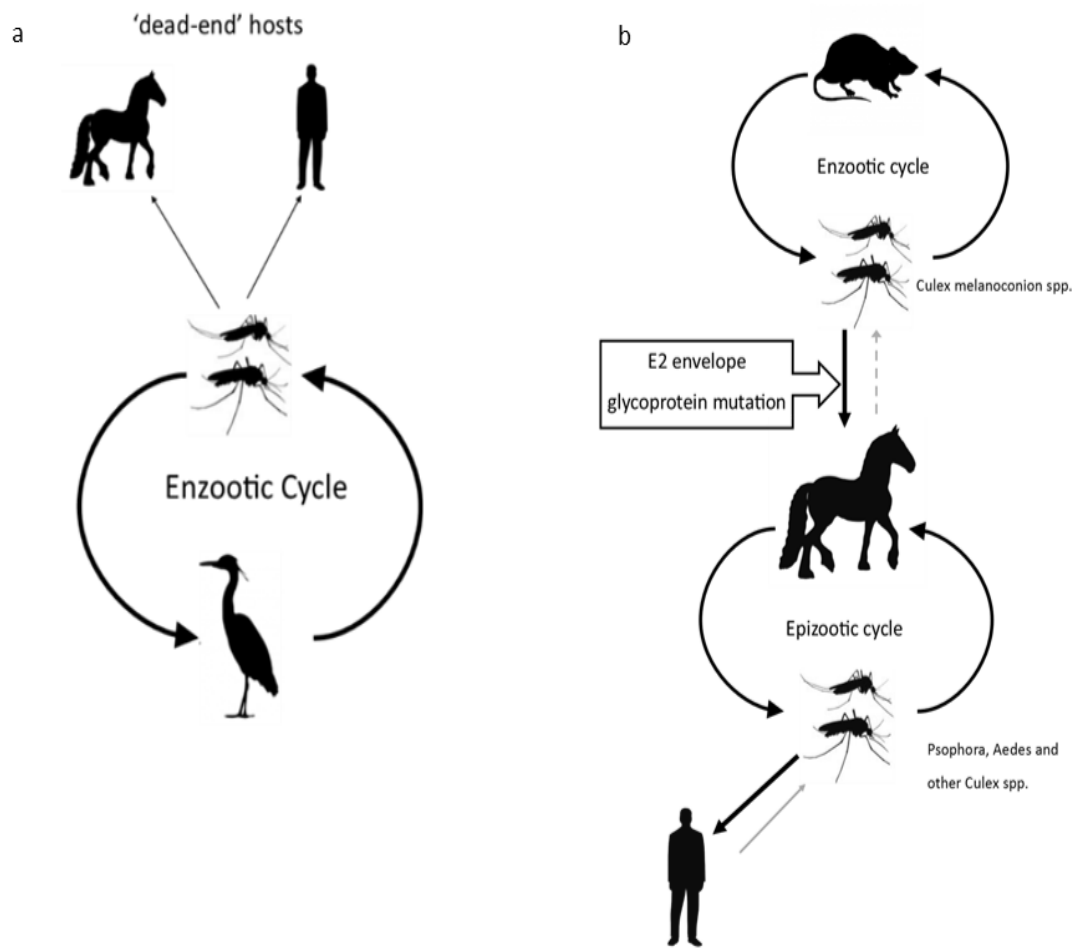

Figure 1.1 Example transmission cycles of equine arboviruses:
(a) Murray Valley encephalitis virus, which has a simple transmission cycle in which the horse is a 'dead-end' host similar to other arboviruses such as West Nile virus; (b) Venezuelan encephalitis virus.

West Nile virus has the most widespread geographical distribution (Figure 1.2) and the largest known vector and host range of all mosquito-borne flaviviruses (Pradier et al., 2012). In contrast, JEV and MVEV have more restricted ranges. However, it is not clear whether host or vector range may be more important in restricting their distribution.

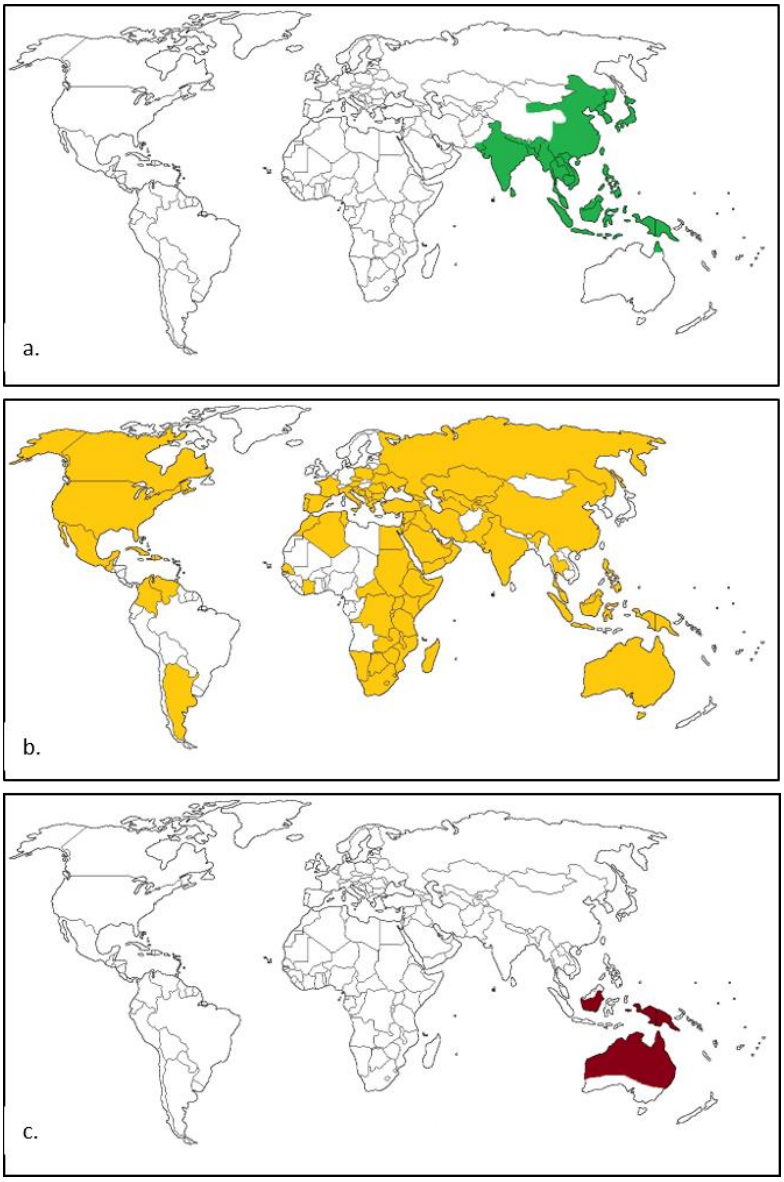

Figure 1.2 Global distribution, by country, of equine flaviviruses:

(a) Japanese encephalitis virus (Impoinvil et al., 2013; Mackenzie et al., 2004); (b) West Nile virus (Chancey et al., 2015; OIE, 2017; Vieira et al., 2015; Zeller and Schuffenecker, 2004); (c) Murray Valley encephalitis virus (Selvey et al., 2014).

1.2.4 Alphavirus epidemiology and ecology

Although alphaviruses have similar transmission cycles to the flaviviruses, they tend to be more complex. The equine encephalitides (EEE, WEE and VEE) are all restricted to the American continent (Figure 1.3a, 1.3b, 1.3c). Eastern equine encephalitis virus affects horses, swine and humans as dead-end hosts (Weaver, 2005). It was traditionally thought to be maintained in an enzootic cycle between passerine birds and mosquitoes. However rodents are now thought to be epidemiologically significant hosts in South America, and possibly in Florida (Arrigo et al., 2010; Day et al., 1996). The ecology of South American EEEV is poorly understood (Weaver et al., 2012). WEEV is maintained in an enzootic cycle between mosquitoes and birds, from Canada to Argentina (Pages et al., 2009), with horses

and humans affected as dead-end hosts (Long and Gibbs, 2007; Reed et al., 2005). VEEV circulates in enzootic cycles between rodent hosts and mosquito vectors in Mexico, Central and South America (Carrara et al., 2005). The virus is antigenically complex with six antigenic subtypes within which there are antigenic variants. The E2 envelope glycoprotein determines equine viraemia and virulence, and mutations in the E2 gene can cause avirulent strains to be more efficiently amplified in horses. This results in an epizootic cycle during which virus amplification in the horse is sufficient to result in mosquito infection (i.e. the horse is no longer a dead-end host) and this is thought to significantly increase the risk of human infection (Greene et al., 2005). Epizootics of VEEV have generally occurred in South America (Figure 1.3c), although an epizootic occurred in Texas in 1971, affecting an estimated 10% of the equine population in the region and 1,500 equids died (Sudia et al., 1975a; Zehmer et al., 1974). Humans are typically considered dead-end hosts, but there is some recent evidence to suggest that humans may develop high enough VEEV titre to continue epidemic transmission in urban environments (Morrison et al., 2008).

Ross River virus is active each year in most regions of Australia and has also caused epidemics in Papua New Guinea, the Solomon Islands, Fiji, New Caledonia and the Cook Islands (Figure 1.3d). Epidemic polyarthritis due to RRV infection is the most common arboviral disease in humans in Australia (Russell, 2002). The major enzootic cycle of RRV involves members of the macropod (i.e. kangaroo) family as the vertebrate host, although other mammals have been suggested as reservoir hosts (Jacups et al., 2008). There is evidence that both horses and humans are able to infect vectors and at least one outbreak is thought to have occurred due to the movement of an infected person (by aeroplane), resulting in ongoing transmission (Harley et al., 2001; Kay et al., 1987; Mackenzie et al., 1994; Rosen et al., 1981).

Getah virus is found from Eurasia to Australasia (Figure 1.3e). The natural transmission cycle is not well described or studied, although swine are thought to play an important role in amplification (Kumanomido et al., 1988). Getah virus appears to have a wide host range, although the main enzootic cycle is thought to be between mammals (particularly rodents) and mosquitoes, as birds show lower seroprevalence rates (Fukunaga et al., 2000; Kumanomido et al., 1988). Horses produce a high enough viraemic titre during an epidemic to infect mosquitoes and direct horse-to-horse transmission has been demonstrated experimentally, although it is unlikely to be a common mechanism for natural infection (Sentsui and Kono, 1980).

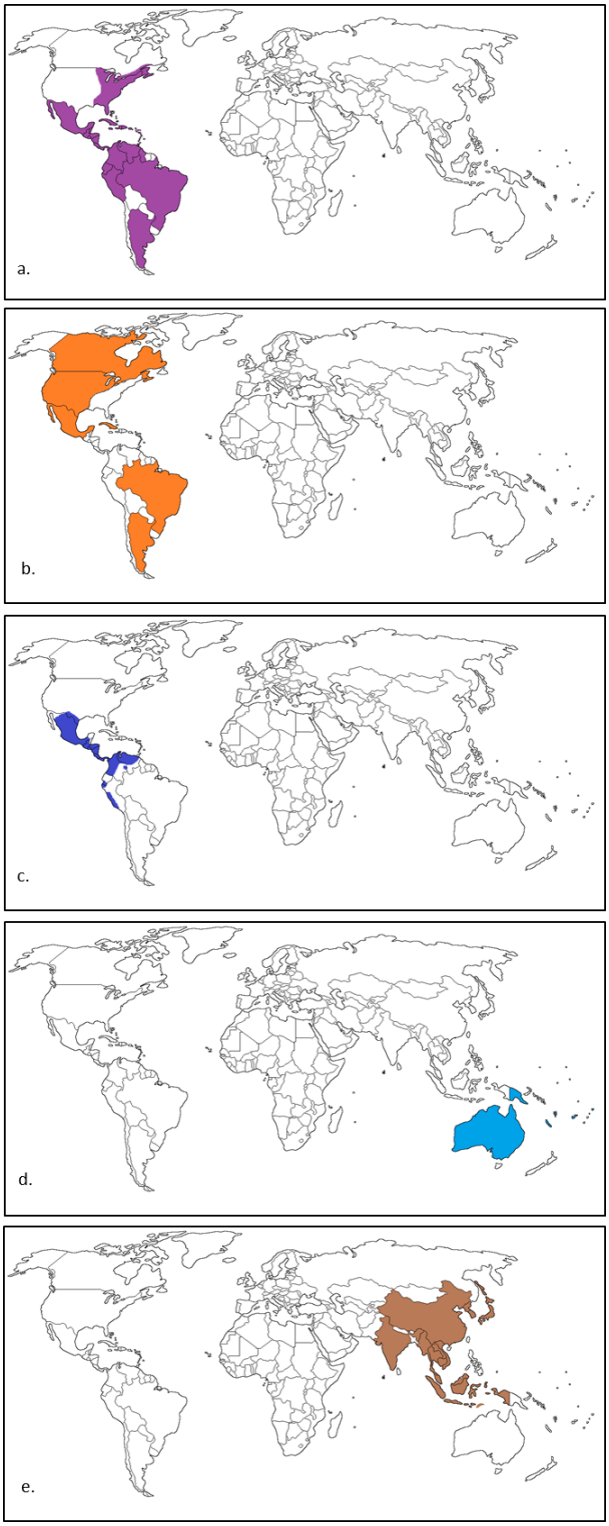

Figure 1.3 Global distribution, by country, of equine alphaviruses:

(a) Eastern equine encephalitis virus (Arrigo et al., 2010; Centers for Disease Control and Prevention (CDC), 2014; Inc and Berger, 2016); (b) Western equine encephalitis virus (Weaver et al., 1997; World Health Organisation, n.d.) (c) Venezuelan equine encephalitis virus (Jiménez et al., 2016; Weaver and Barrett, 2004; Weaver and Reisen, 2010); (d) Ross River virus (Harley et al., 2001; Jacups et al., 2008; Rosen et al., 1981); (e) Getah virus (Fukunaga et al., 2000).

1.2.5 Others

Bunyamwera virus

Since 2013, Bunyamwera virus, an Orthobunyavirus, has emerged as a cause of neurological disease and possibly abortion in horses in Argentina (Tauro et al., 2015, 2016). Clinical signs include apparent disorientation, weakness, visual deficits, tongue protrusion, recumbency and death. Transmission cycles are poorly characterised, although mammals are considered to be amplifying hosts and most isolates have been recovered from mosquitoes. Seroprevalence among birds in Argentina indicate that they could be in involved as endemic hosts (Tauro et al., 2009).

Several other viruses cause occasional clinical disease in horses (Table 1.1) (Attoui et al., 2009; Bertone et al., 2004; Hubálek et al., 2014).

Arbovirus emergence

In order to understand the potential impacts of globalization and climate change on the disease patterns of equine arboviral disease, knowledge of the complexities of transmission cycles, vector life cycle and the effects of climate change on the vector and vector infection dynamics are vital. Due to the involvement of multiple different host species and vectors for some of the equine arboviruses, risk prediction for epidemic transmission and for virus establishment and endemicity is challenging. Even for arboviral diseases with two-host transmission cycles such as African horse sickness, complex models are required to investigate the risk of virus introduction leading to autochthonous transmission (Faverjon, 2015), let alone persistence or establishment in a new region. For non-endemic countries including the UK there is a lack of information on the presence of vectors for the viruses discussed, their vectorial capacity, and the competence of potential reservoir hosts, which may not be present (and therefore studied) in endemic areas.

1.3.1 Virus introduction

Mosquito-borne equine pathogens may theoretically enter the UK through several means, and these are summarised in Table 1.4 for the viruses discussed in this thesis.

Introduction Pathway		WNV	JEV	EEEV	WEEV	VEEV	MVEV	RRV	Getah Virus[1]
Vector	Adult	✓	✓	✓	✓	✓	✓	✓	✓
	Eggs		✓[2]				?[a,3]	✓[4]	?
Wildlife	Birds	✓	✓	✓	✓		✓		
Livestock	Large animals		Swine[5]			Equids[5]	Equids?[15]		
	Macropods							✓	
Exotic Pets	Birds	✓	✓[5]	✓[5]	✓[5]		✓		
	Small Mammals	✓	✓[5]	✓[5]	✓[8]	✓		?[16,17]	?
	Amphibians/Reptiles	✓	✓[6,7]	✓[5]	✓[9]				
	Primates					✓			
Pet transport	Dogs					✓[10,11,12,13]			?
Human Transport						✓[14]		✓[18]	?

Table 1.4 Potential modes of introduction of virus into the UK.

a – No evidence of ongoing transmission after vertical transmission in vector

1. (Fukunaga et al., 2000)
2. (Takashima and Rosen, 1989)
3. (Marks, 1967)
4. (Harley et al., 2001)
5. (Durand et al., 2013)
6. (Oya et al., 1983a)
7. (Oya et al., 1983b)
8. (Hardy et al., 1974)
9. (Gebhardt and Hill, 1960)
10. (Bivin et al., 1967)
11. (Davis et al., 1966)
12. (Dickerman et al., 1973)
13. (Taber et al., 1965)
14. (Adams et al., 2012)
15. (Kay et al., 1987)
16. (Jacups et al., 2008)
17. (Russell, 2002)
18. (Lau et al., 2017)

The initial arrival of virus into a new region is sometimes termed 'virus release' (Faverjon, 2015), however in this thesis the term 'virus introduction' is used to mean the arrival of infectious virus on UK shores.

JEV

JEV could be introduced into the UK or Europe through transport of birds or pigs, or by migratory birds, although migration patterns of waterbirds are in general north to south (Si et al., 2009), so direct introduction to Europe by migratory birds is unlikely. One of the main reservoir species, the Black-crowned Night Heron (*Nycticorax nycticorax*) is a summer visitor to Europe including France (Lebarbenchon et al., 2007; Ledwoń and Betleja, 2015). These birds are migratory and may therefore, over time, contribute to dissemination from Asia into Europe. Bird migration in relation to emerging infectious disease is a current topic generating research interest (Thomas et al., 2008). Cross-boundary transport of pigs is well controlled, so the most likely mode of entry may be the trade and transport of birds. JEV RNA has been found in *Culex pipiens* mosquitoes in Italy, (Ravanini et al., 2012) although this has not yet been repeated.

WNV

Introduction of WNV into new areas is generally thought to occur through bird migration, although the significance of dispersal through infected mosquitoes is unknown (Rizzoli et al., 2015). Importation through anthropogenic means is also possible (Table 1.4). It may be the case that other related flavivirus infections such as Usutu virus or WNV strains of low pathogenicity for birds and for humans and horses, confer cross-protection and it has been suggested that the low number of human cases of WNV in Spain, despite high seroprevalence in birds, may be a result of this (Beck et al., 2013; Rizzoli et al., 2015). This could also slow the expansion of highly pathogenic strains of WNV in Europe. However, Rizzoli et al. (2015) suggest that due to the cross-protection of birds, surveillance based on analysis of dead birds may fail to detect ongoing circulation and that active monitoring of sentinel or wild birds and horses is preferable.

MVEV

Importation of waterbirds from Australia represents a theoretical risk for virus introduction into Europe, and modest numbers of exotic birds are imported into Europe from Australia (Durand et al., 2013). Vertical transmission of virus in *Aedes* species with desiccation resistant eggs has been demonstrated, by identification of virus in *Aedes tremulus* males (Broom et al., 2003). Vertical transmission in drought resistant eggs can have important implications for

virus introduction particularly in container breeding species such as *Aedes tremulus*, which is known to be a nuisance biter of humans (Marks, 1967). However, there is no direct evidence of transmission ability of this species. Overall, introduction risk of MVEV into the UK or Europe is considered low, compared to the other arboviruses discussed.

EEEV

In relation to the threat to Europe and the UK in particular, the high level of imports of reptiles and rodents into the EU as exotic pets from North America (8459 consignments between 2005 and 2009, from widely distributed locations) represents a potential risk for virus introduction (Durand et al., 2013). Imported birds also represent an import risk.

WEEV

Nestling passerines are considered the main amplification host and rodents, reptiles and amphibians have been found serologically positive to WEEV (Hardy et al., 1974; Long and Gibbs, 2007). Therefore, imported birds and exotic pet species such as garter snakes and tortoises from parts of the Americas should be considered a risk. Garter snakes have been found to be naturally infected (Burton et al., 1966) including *Thamnophis sirtalis* which has been a popular pet species. It has been shown experimentally that the garter snake is a likely overwintering host for WEEV (Gebhardt et al., 1964; Gebhardt and Hill, 1960; Thomas et al., 1960; Thomas and Eklund, 1962). It has also been demonstrated that some South American snakes can maintain viraemia for 2-3 weeks (Gebhardt et al., 1964 citing Rosenbusch, 1939). Other pet species such as the Texas tortoise have also been found to be infected in nature (Bowen, 1977).

VEEV

Human and equine transport, and possibly transport of pet dogs constitute an import risk, as well as exotic rodent imports (Table 1.4). Because horses are major amplification hosts, epidemic transmission may be possible if British mosquitoes can act as epidemic vectors.

RRV

Importation of macropods, horses and possibly small mammals constitute potential risks for importation of RRV. Human air travel has been associated with virus introduction and ongoing transmission without the presence of macropods (Lau et al., 2017). Human beings produce high viraemic titres (up to $10^{6.3}$ ID_{50}/ml) and are thought to be efficient amplifiers (Rosen et al., 1981). Rates of clinical to subclinical infections in humans have been reported from 1:0.3 to as high as 1:80 (Russell, 2002) and the large number of human cases in

Australia each year would suggest that human dissemination of Ross River virus is an ongoing risk. Field and laboratory data suggest that vertical transmission occurs in the major *Aedes* vectors of RRV and the container breeder *Aedes tremulus* (Harley et al., 2001), therefore transport of infected desiccation resistant eggs is a potential mode of importation.

GETAH VIRUS

Because the natural transmission cycle of Getah virus is not well described, introduction potential is unclear. However, importation of horses, potentially rodents, and theoretically swine (although movements are restricted due to other disease risks), are possible modes of importation (Fukunaga et al., 2000; Kumanomido et al., 1982, 1986; Sentsui and Kono, 1980).

1.3.2 Virus Persistence and Regional Dissemination

In this section general discussion and examples are presented. Discussion of individual viruses with specific reference to vector species, candidate vector species, and reservoir host species present in the UK follows.

It is important to remember that equine and human movement is not a risk (or is low risk) for many of these diseases, as they are 'dead-end' hosts. Therefore, disease introduction may occur without risk of onward transmission of virus, due to the absence of, or low viral titre in the blood at the time of importation, for example the case of clinical West Nile encephalitis in a horse imported into the UK (Fooks et al., 2014). If local vectors are infected, then clinical cases in horses or humans may occur. Occurrence of a small number of autochthonous (locally-acquired) infections could occur without local reservoir host involvement, where all vectors are infected from imported hosts. This type of locally-acquired infection may occur without being detected as many infections with equine arboviruses are subclinical. In certain cases, a small number of onward transmission events could occur without a vector contacting the importation host, for example by blood transfusion (Harvala et al., 2009). It has also been suggested that bird-to-bird transmission of WNV may occur in some species and contribute to overwintering of the virus (Ip et al., 2014). A competent vector, however, is required for epizootic spread. Ongoing transmission in the case of a VEEV epizootic strain or RRV would require only susceptible equines or humans and competent vectors, and in this case equines could represent a public health risk. In the case of other equine mosquito-borne pathogens, competent local reservoir hosts are required for ongoing transmission, as with the introduction of WNV into the USA.

Steps required for virus introduction, dissemination and persistence are summarised in Figure 1.4 (virus introduction in an infected host) and Figure 1.5 (virus introduction in an infected vector).

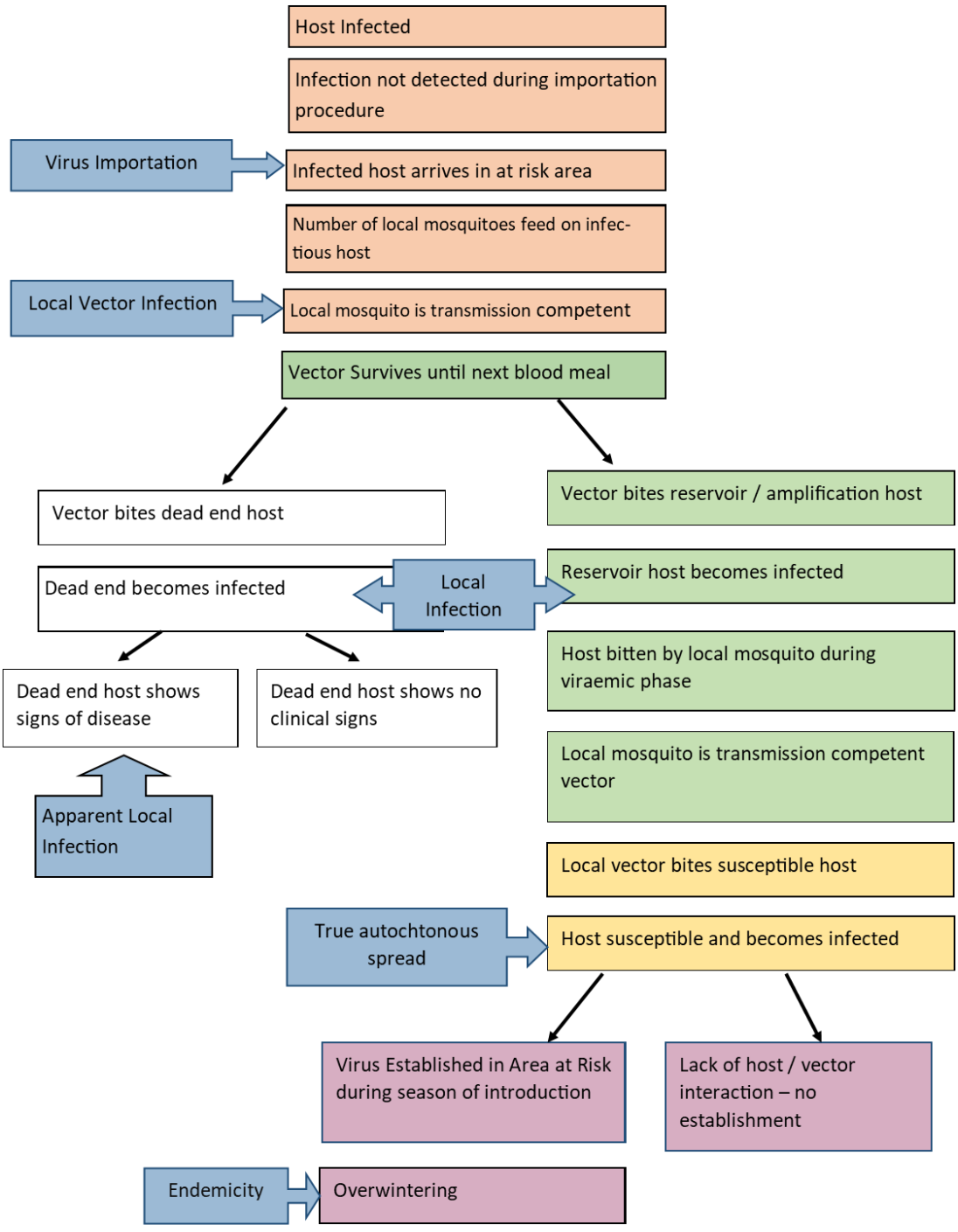

Figure 1.4 Consequences of virus introduction in an infected host.

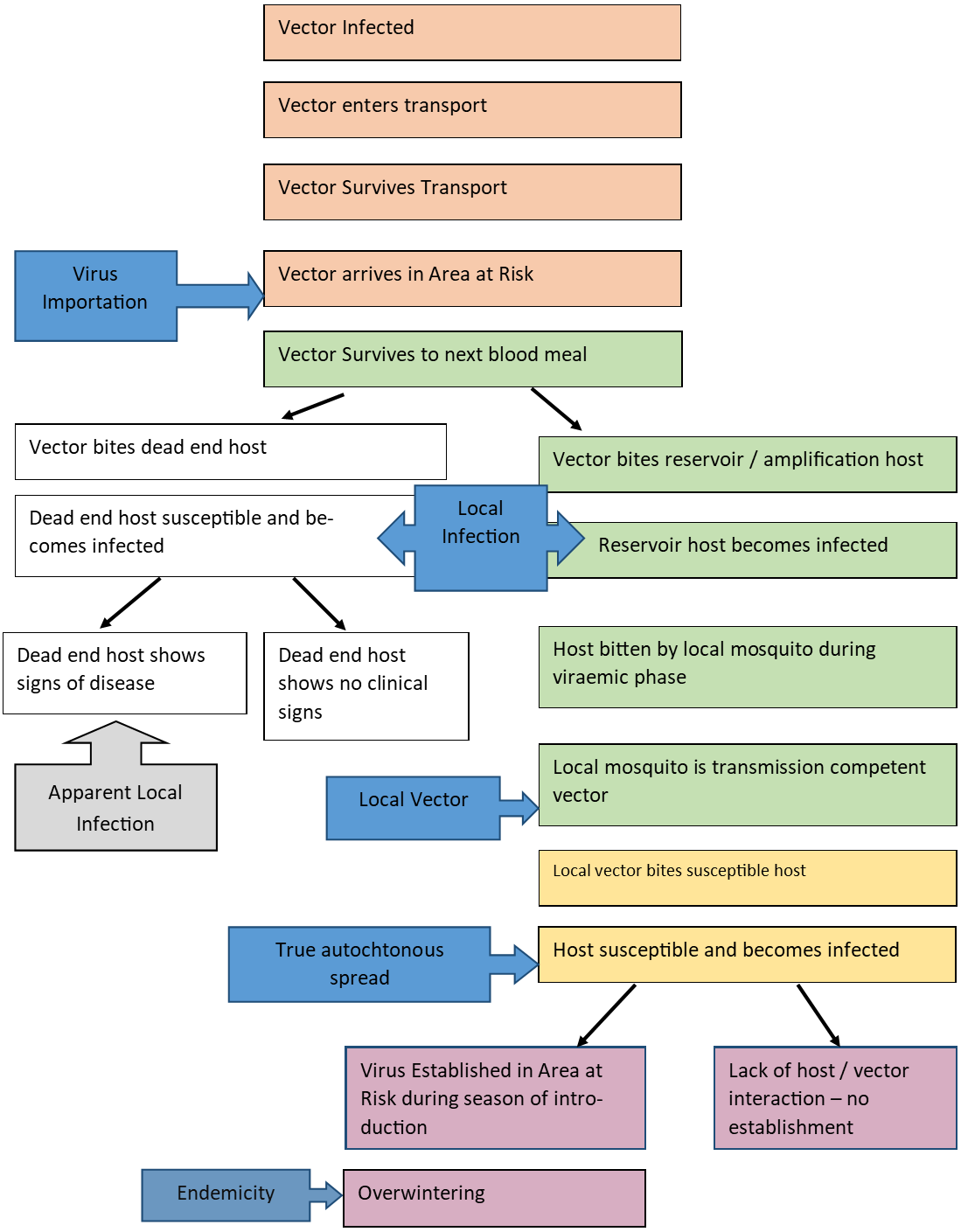

Figure 1.5 Consequences of virus introduction in an infected vector.

VECTOR PRESENCE

The distribution of equine arboviruses is dependent on the presence of competent vectors. The presence of an alternative species to the main known vector species can have a profound impact on the epidemiology of incursion, but this is difficult to predict. For example, the main insect vector of JEV in Asia is *Culex tritaeniorhynchus*, which can be infected when there are very low levels of viraemia in the host. However, when there was an incursion of JEV in northern Australia, the main vector was *Culex annulirostris* (Hanna et al., 1996; van

den Hurk et al., 2012), which is thought to require higher titres to become infected. It is not clear whether JEV failed to become endemic in northern Australia for this reason or due to the presence of other flaviviruses conferring cross-protection to hosts, or because *Cx. annulirostris* preferentially feeds on marsupials and not wading birds or pigs (Van Den Hurk et al., 2010).

For some other viruses, such as EEEV, it is not clear whether virus introduction into the UK or Europe would find adequate populations of competent vectors for ongoing transmission. Of the major vectors of EEEV in the Americas, only *Cs. morsitans* is present in Europe (Gaffigan et al., 2017). Without further information such as vector competence and ecological information on vector populations and host–vector interaction, it is not possible to accurately predict whether mosquito populations present in the UK would be capable of facilitating epidemic or endemic transmission.

INVASIVE VECTOR INTRODUCTION

The risk of arbovirus vector introduction is important in both endemic and non-endemic regions. Invasive vectors, i.e. introduced species that have increased in number and regional range (Juliano and Lounibos, 2005), include vectors of equine arboviruses such as *Aedes albopictus, Culex pipiens* and *Culex quinquefasciatus* (Juliano and Lounibos, 2005). The introduction of an invasive vector with high vector competence could increase the risks posed by virus introduction, or even directly facilitate virus introduction. Surveillance for invasive mosquito species is therefore carried out by Public Health England (Vaux and Medlock, 2015).

Introduction may occur through human activity such as the movement of people, livestock or goods. The used tyre trade has been implicated in long distance sea transport of mosquito eggs of *Aedes* mosquitoes (Hurlbut, 1973) and desiccation-resistant eggs are associated with increased probability of species introduction (Juliano and Lounibos, 2005). Mathematical modelling applied to assess the risk of WNV-infected mosquitoes being introduced to the UK from America led to the conclusion that each summer there is a high risk of at least one WNV-infected mosquito arriving at Heathrow airport, which has a moderate density of susceptible vector and bird species that could allow local dissemination of the virus (Brown et al., 2012). The airport is also in a region with a high density of horses (Boden et al., 2012).

VECTOR-HOST INTERACTION

The details of vector-host interaction are complex and vary depending on the ecosystem. For some of the equine arboviral diseases, such as GETV, Bunyamwera and to an extent EEEV

in South America, they have not been well studied. This lack of knowledge creates major challenges in predicting the outcome of an arboviral disease outbreak in a non-endemic region.

Apart from epidemic VEEV, in which the amplification host is the equid and possibly RRV (Lau et al., 2017), multiple hosts are necessary for completion of the transmission cycle of the arboviral diseases described here. For most of these viruses, vectors that blood-feed from both birds or small mammals and large mammals are therefore required for equine infection to occur. Vectors that bite multiple host types (usually avian and mammalian) are commonly referred to as 'bridge vectors'. Some of these bridge vectors are indiscriminate ('catholic') feeders whereas others mainly feed on avian species, and occasionally feed on large mammals including horses, or on humans. This can therefore lead to infection in these species. Examples of bridge vectors are *Culex pipiens* for WNV (Hamer et al., 2009) or *Culiseta melanura* for EEEV (Armstrong and Andreadis, 2010), both of which cause infections in horses and humans.

CANDIDATE BRITISH VECTOR SPECIES

British mosquito species that have the potential to become involved in transmission in the event of WNV are reviewed by Medlock et al. (2005), although *Culex modestus* should now be added to that list. Further ecological information regarding mosquito species present in the UK is reviewed by Becker et al. (2010) and ecological information focussed on British populations is summarised in Table 1.5. Information regarding potential for host vector interaction, and vector status is summarised in Table 1.6. The specific potential for mosquito-horse interaction in the UK has not been studied, and that is one of the aims of this thesis.

Species	Breeding habitats [1,2,3,4]	Annual generations[1,2]	Adult activity[1,2,3]											
			Jan	Feb	Mar	Apri	May	June	July	Aug	Sep	Oct	Nov	Dec
Aedes cinereus / geminus	Su+ Flooded grassland, shallow pools, ponds, ditches, marshes	1						A	A	A +				
Anopheles claviger	Sa+/- Su- Cool permanent water, natural and artificial	2			A	A	A +	A +	A +	A +	A +			
Anopheles plumbeus	Tree holes - occ containers, diff to find -v sens vibration	2				A	A	A	A	A	A	A		
Anopheles maculipennis s.l.	Sa+/- various habitats	?	H	H	H/ A	A	A	A	A ++	A ++	A ++	H/ A	H	H
Coquilletidia richiardii	Difficult to find, various permanent sites	1 Au				?	A	A	P	A	A			
Culiseta annulata	Wide variety Sa+/- Su-, likes manure, natural and artificial	M	H	H	H	H	A	A ++	A ++	A	H	H	H	H
Culiseta litorea	Variety of sites, Sa+/- Su+/-	1					A	P	P	A	A			
Culiseta. morsitans	Sa+/- Su+/- Wide variety, usually but not always permanent	1				A	A	A	A	A	A	A		
*Culiseta subochrea**	Wide variety, Sa+/-	M Au	H	H	H	H	A	A	A	A	H	H	H	H
Ochlerotatus annulipes	Su+ or partially shaded swamps and pools	1				A	A +	A +	A +	A +	A			
Ochlerotatus cantans	Su-, Sa-, ditches, pools etc	1				A	A	A	A ++	A	A			
Ochlerotatus caspius	Sa+/- Areas of intermittent flooding	M				A	A	A	A	A	A	A		
Ochlerotatus detritus	Coastal marsh	M			A	A	A	A	A	A +	A ++	A +	A	
Ochlerotatus dorsalis	Su+ or partial shade Sa+/- intermittent pools	M					A	A	A	A	A			
Ochlerotatus. flavescens	Su+ Mud / plant debris - brackish marshes^	1-2					A	A	A					
Ochlerotatus punctor	Temporary pools, woodland mainly	1				A	A	A	A	A	A	A		
Ochlerotatus rusticus	Woodland pools and ditches	1				A	A ++	A ++	A	?				
Ochlerotatus geniculatus	Tree holes	M?				A	A	A	A ++	A ++	A			
Culex modestus	Sa+/- marshes, irrigation channels, ponds	1							A +	A +	A +			
Culex pipiens s.l.	Variety of artificial and natural sites	M Au^{ml}	A ml /H	A^{ml} /H	A^{ml} /H	A	A	A	A	A	A	A	A	A^{ml} /H
Culex torrentium	Variety of artificial and natural sites, including tree holes	M				A	A	A	A	A	A	A	A	
Aedes vexans	Flooded grassland	M^					A	A	A	A				

Table 1.5 Relevant ecological information regarding the less rare British mosquito species.

A - adults active; A+ - large numbers of active adults; A++ - peak adult activity; H - hibernating adults; M – multivoltine; ml - *Culex pipiens molestus;* Sa+ - saline water; Sa- - freshwater; Su+ - favours sunlit sites; Su- - favours shaded sites; ^ - no UK specific information; * - little information - believed to be similar to *Cs. annulata*

1. (Medlock et al., 2005)
2. (Becker et al., 2010)
3. (Cranston et al., 1987)
4. (Snow, 1991)

Species	Distribution[1,2,3]	Dispersal distances[4,5,6]	Biting Habits[4,5,7]	Host Biting[1,8]	Vector Status[8]	Candidate British Vector	
						AH	MH
Ae. cinereus / geminus	Patchy	500m	Outdoors, in wooded areas, peaks at dusk and dawn	M, B, E	EEEV [I]	WNV[1,] MVEV WEEV	All
An. claviger	Very abundant - fens		Open areas	M, E			
An. plumbeus	Widespread		Indoors / outdoors, peak at sunset	M, B, E	WNV [L]	WNV[1]	
An. maculipennis s.l.	Moderate numbers - fens	>2 miles	Indoors and outdoors, at night ^	M,B,E	WNV [I]		
Cq. richiardii	Very abundant - fens		Indoors and outdoors, peak just after sunset	M,B,E	WNV [I]	WNV[1] MVEV WEEV	All
Cs. annulata	Very abundant - fens	>2 miles	Indoors and outdoors, day and night^	M,B,E	WNV [L]	WNV[1] MVEV WEEV	All
Cs. litorea	Widespread in south			M,B		WNV[1] MVEV WEEV	All
Cs. morsitans	Widespread	>2 miles	At night^	B,M	EEEV [Z]	WNV[1] MVEV WEEV	All
*Cs. subochrea**	Uncommon		Prefers to bite outdoors, during the day^	M, E			All
Oc. annulipes	Abundant - fens		Bites during the day, peak at dusk^	M,E,(B)			All
Oc. cantans	Abundant - fens	> 400m	Open areas at dawn and dusk, wooded areas in daytime	M,B,E		WNV[1] MVEV WEEV	All
Oc. caspius	Common coastal	>5 miles	Open areas, during the day more than at night	M,B,E	WNV [I L]	WNV MVEV WEEV	All
Oc. detritus	Widespread, patchy	>5 miles	Open areas, peaks 9-10am and at dusk	M,B,E	WNV [L] JEV [L]	WNV[1] MVEV WEEV	All
Oc dorsalis	Widespread patchy, locally abundant	>5 miles	Open areas, peak before sunset	M,E	WEEV [I L]	WNV[1] MVEV WEEV	All
Oc. flavescens	Uncommon, patchy		Open areas, peaks at dusk and dawn	M,E			All
Oc. punctor	Widspread, very localised	>2 miles	Outdoors in shade, peak 2-3 hours before sunset	M,B,E	WNV [L]	WNV[1] MVEV WEEV	All
Oc. rusticus	Widespread, patchy		Wooded areas, peak at dusk	M,E,(B)			All
Oc. geniculatus	Patchy, locally abundant	<2km	Open areas at dusk, shady areas in daytime^	M,E			All
Cx. modestus	Kent, Essex		Bite during the day in open areas - peak at dawn^	B,M,E	WNV [V L]	WNV[1] JEV	JEV
Cx. pipiens s.l.	Common, widespread, abundant		At night ^	B (M,E)	WNV [V L] JEV [L] EEEV[N] WEEV [N] VEEV [N]	WNV	JEV
Cx. torrentium	Abundant			B (M)		WNV*	JEV*
Ae. vexans	Rare	> 15km		M,B,E	WNV [I] EEEV [IL]	WNV	All

Table 1.6 Host biting, vector and candidate vector status of the less rare British mosquito species.

AH - Viruses with majority avian amplifying hosts: WNV, MVEV, WEEV; MH - Viruses with mammalian amplifying hosts: JEV, EEEV, VEEV, RRV, Getah virus; * - Enzootic vector only A- amphibians; B – birds; M – mammals; R – reptiles; L – Laboratory competent vector; I – Implicated in disease transmission worldwide; N – Non-competent as laboratory vector; V – Ecologically sign significant bridge vector worldwide; Z – Ecologically significant enzootic vector worldwide; ^ - no UK specific information

1. (Cranston et al., 1987)
2. (Snow, 1991)
3. (Becker et al., 2010)
4. (Service, 1969)
5. (Snow and Medlock, 2008)
6. (Balenghien et al., 2006)
7. (Service, 1971a)
8. (Chapman et al., 2016)

1.3.3 Potential for Viral Dissemination and Persistence in the UK

In this section individual viruses are discussed, with regards to the potential for persistence and dissemination in the UK.

JEV

VECTORS

Cx. pipiens pallens and *Cx. tritaeniorhynchus* are the main vectors in Asia, although *Cx. annulirostris* is the main vector in Australia, and *Cx. gelidus* is important in transmission involving pigs in Malaysia (Gresser et al., 1958; Impoinvil et al., 2013). *Cx. vishnu* and *Cx. quinquefasciatus*, and *Ae.j.japonicus*, an invasive species, among others, are also implicated as JEV vectors (Rosen, 1986; Sucharit et al., 1989). *Culex tritaeniorhynchus*, was found in Greece in 2003 (Samanidou and Harbach, 2003).

BRITISH CANDIDATE VECTORS

None of the known ecologically significant vectors of JEV are present in the UK. The British mosquito *Ochlerotatus detritus* has been shown to be a competent laboratory vector (MacKenzie-Impoinvil et al., 2014). *Culex pipiens s.l.* and *Culex torrentium* are candidate vectors which should be tested for vector competence for JEV.

ONGOING TRANSMISSION POTENTIAL

Species of heron known to be involved in the enzootic cycle in Asia, are present in the UK, such as *Egretta garzetta*. Only a selected number of species of birds have been competence tested in the laboratory for ability to infect mosquito hosts. Other birds found to produce viraemia high enough to infect *Cx. tritaeniorhynchus*, and present in the UK include the Rock Pigeon (*Columba livia*), the European Starling *(Sturnus vulgaris)*, the Mallard (*Anas platyrhynchos*), the House Sparrow *(Passer domesticus*), and the Cattle Egret (*Bubulcus ibis)* (Nemeth et al., 2012).

WNV

VECTORS

WNV is primarily transmitted by *Culex* mosquitoes, including *Cx. pipiens*, *Cx. univittatus*, *Cx. antennatus*, *Cx. vishnu* complex in India, and *Cx. annulirostris* in Australia. Part of the success of mosquitoes such as *Cx. pipiens* and *Cx. quinquefasciatus* as vectors may be due to their use of peri-domestic containers as breeding sites. Amplification of WNV early in the transmission season may involve ornithophilic vectors in enzootic transmission, before spill-

over occurs into mammals due to infection of vectors with more catholic feeding habits (Petersen, 2015).

VECTOR SPECIES PRESENT IN THE UK

Cx. pipiens are widespread throughout the UK. *Cx. modestus* has been identified in Kent and Essex, and laboratory vector competency of European populations has been shown (Balenghien et al., 2007). Other UK species which are less common, such as *Aedes vexans* (Turell et al., 2005), *Ochlerotatus punctor, Ochlerotatus geniculatus* and *Anopheles plumbeus* (Vermeil et al., 1960) have been shown to be laboratory competent in Europe or America. All these species have been demonstrated to feed on horses in the UK with the exception of *Ochlerotatus geniculatus.*

BRITISH CANDIDATE VECTORS

Medlock et al., (2005) proposed as candidate bridge vectors: *Anopheles plumbeus, Ochlerotatus caspius, Ochlerotatus detritus, Ochlerotatus dorsalis, Coquillettidia richiardii, Culiseta annulata, Culiseta morsitans, Aedes cinereus, Ochlerotatus cantans* and *Ochlerotatus punctor*. However since this time, *Ochlerotatus caspius* has been shown to be a poor laboratory vector (Balenghien et al., 2008) and British *Oc. detritus* has been demonstrated to be laboratory vector competent (Blagrove et al., 2016). *Coquillettidia richiardii* appears as a principal European vector in the literature (Higgs et al., 2004; Hubálek and Halouzka, 1999; Medlock et al., 2005; Savage et al., 1999); however primary evidence for this is lacking.

ONGOING TRANSMISSION POTENTIAL

The reservoir competence for many European bird species is unknown, as very little research has been undertaken on European species (Muñoz et al., 2012), compared to those native to the US. It is known that the House Sparrow (*Passer domesticus*) which is present in the UK albeit in much lower numbers than historically, are frequently infected with WNV in America and are accepted to serve as important amplifying hosts in some areas (Hayes et al., 2005; Komar et al., 2005). The European Starling (*Sturnus vulgaris*) and Carrion Crow *(Corvus corone*) (Rizzoli et al., 2015) are competent hosts.

MVEV

VECTORS

Culex annulirostris is the most significant vector in Australia (Boyle et al., 1983), although other *Culex* and *Aedes* spp. may also be involved in transmission.

British candidate vectors

There are no studies on vector competence in European mosquito species. Therefore, *Culex pipiens pipiens,* and *Culex torrentium,* which are not present in Australia (Russell, 2012) are prime candidates for laboratory vector competence testing.

Ongoing Transmission Potential

The most studied enzootic reservoirs of MVEV *Nycticorax caledonicus* and *Egretta intermedia* are not present in Europe, although other *Ciconiiformes* are thought capable of producing viraemias which can infect vectors. The role of mammals in virus transmission in unclear, and the current distribution of the virus is closely associated with that of *Nycticorax caledonicus.* Significant ongoing transmission of MVEV in the UK, therefore, seems unlikely.

EEEV

Vectors

Enzootic circulation is chiefly between *Culiseta melanura,* or *Culiseta morsitans* and passerine birds. *Aedes, Ochlerotatus* and *Coquillettidia* species with a broad host range act as bridge vectors. In South America the main vectors are mosquitoes belonging to the genera *Culex* and *Aedes* (Oliveira et al., 2014). Detail of mosquito vector species involved is not available and in general the ecology of South American EEEV requires further study, as is it is still poorly understood (Weaver et al., 2012).

Vector species present in the UK

Culiseta morsitans is widespread throughout the UK, has often been found to be naturally infected in America (Molaei et al., 2013), and is implicated as an ecologically significant bridge vector (Andreadis et al., 1998; Armstrong and Andreadis, 2010; Centers for Disease Control and Prevention (CDC), 2006). Andreadis et al., (1998) assert that the enzootic transmission capacity of *Cs. morsitans* rivals that for *Cs. melanura*, except that numbers decline from mid-August (in Connecticut). Information on laboratory transmission is not available. *Cs. morsitans* has been shown to bite horses in Europe (Börstler et al., 2016). *Aedes cinereus* is a suspected vector based on virus isolation by Armstrong and Andreadis (2010), although laboratory vector competence has not been tested.

British candidate vectors

With reference to the known vector status of *Culiseta* species in North America for EEEV, the widespread British species *Culiseta annulata* is a candidate vector which should be

tested for laboratory vector competence. Pages et al. (2009) surmise that in Europe *Aedes vexans* and other *Aedes* species may play a role, in the event of virus introduction. Armstrong and Andreadis (2010) assert that *Ae. vexans* is a poorly competent for EEEV, however results vary according to study methodology and, probably, strain of virus or mosquito population (Chamberlain et al., 1954; Davis, 1940; Vaidyanathan et al., 1997). Armstrong and Andreadis (2010) further comment that *Ae. vexans* is unlikely to be ecologically significant in North America, and indeed *Ae. vexans* is not considered ornithophilic. Based on these studies, *Ae. vexans* cannot be ruled out as a candidate vector but is unlikely to have a large ecological role unless population dynamics change, as it is currently rare in the UK (Medlock, 2005). Pages et al. (2009) describe *Culex pipiens s.l.* as a "ubiquitous vector" with potential for involvement in the enzootic cycle. However, laboratory studies have not found the species to be competent for transmission of EEEV. Although the maximum extrinsic incubation period tested was only 14 days, there was also no virus dissemination identified at this time point (Sardelis et al., 2002). Davis (1940) utilised multiple species of infective hosts, and time points up to 30 days were included, providing the most convincing effort to rule out transmission by *Cx. pipiens*. In fact members of the *Cx. pipiens* complex which have been tested have been uniformly non-competent for transmission of EEEV (Aviles et al., 1990), WEEV (Hammon and Reeves, 1943; Merrill et al., 1934a) and VEEV (Aviles et al., 1990; Hammon and Reeves, 1943; Turell et al., 2003).

In relation to invasive mosquitoes present in Europe, *Aedes albopictus* has been found naturally infected and is a proven laboratory vector (Turell et al., 1994), but has not been implicated in transmission in America. *Ochlerotatus japonicus japonicus* is another invasive species present in Europe and a competent laboratory vector (Sardelis et al., 2002). Its catholic feeding habits make it a candidate bridge vector of EEEV.

Ongoing Transmission Potential

Competent avian hosts are certainly present in the UK and Europe, for example the House Sparrow (*Passer domesticus*) and Starling (*Sturnus Vulgaris*) (Arrigo et al., 2010; Komar et al., 1999), both of which have been shown to be competent in the US. EEEV may be transmitted between birds, for example by pecking (Satriano et al., 1958) and by chicken mites (Durden et al., 1993) at least under experimental conditions. Transmission between birds may increase the likelihood of a competent mosquito vector contacting an infected host in a previously unaffected geographical location. The availability of non-avian reservoir hosts in the UK is unknown, however, there are both known vector species, and known

reservoir hosts in the UK and therefore virus introduction presents a risk of ongoing transmission.

WEEV

VECTORS

Culex tarsalis is considered the main vector in North America. Maintenance vectors in South America are unknown. As the summer season progresses bridge vectors become infected, and spill-over into mammals and possibly reptiles and amphibians occurs. An *Aedes (Ochlerotatus) dorsalis* – rabbit cycle has been described (Fulhorst et al., 1994; Weaver et al., 1999; Weaver, 2005).

VECTOR SPECIES PRESENT IN THE UK

Ochlerotatus dorsalis, a competent laboratory vector (Kramer et al., 1998) is present in the UK and has been implicated in a natural transmission cycles in the Americas (Fulhorst et al., 1994; Hayes et al., 2005; Spalatin et al., 1963; Zacks and Paessler, 2010), and vertical transmission in *Oc. dorsalis* occurs, potentially contributing to overwintering (Fulhorst et al., 1994). Isolations of WEEV have also been made from *Aedes vexans* (Hayes et al., 1976; Sekla et al., 1980) and *Oc. flavescens* (Spalatin et al., 1963) which have been implicated in transmission in the Americas (McLintock et al. 1970).

BRITISH CANDIDATE VECTORS

Other *Ochlerotatus spp.* are potential candidates. *Culex pipiens pipiens* from Argentina were found to not be competent laboratory vectors (Aviles et al., 1990) although other members of the *Cx. pipiens* complex, *Cx. pipiens pallens* and *Cx. pipiens* quinquefasciatus were found to be competent laboratory vectors of WEEV in one study (Wang ZhongMing et al., 2012). Therefore, *Cx. torrentium* should be investigated for laboratory vector competency, as a candidate enzootic vector.

ONGOING TRANSMISSION POTENTIAL

Antibodies to WEEV have been found in both the Eastern grey squirrel in the US (Amin and Thompson, 1974) which is the species present in the UK, and the Western grey squirrel (Browne et al., 1956) which was shown to produce significant viraemia after experimental inoculation, but again WEEV strain was important (Hardy et al., 1974), and level of viraemia from natural infection has not been studied to the author's knowledge. Hardy et al. (1974) also reported on 5 other species of American rodents which were able to produce sustained viraemias. Passerine birds which could act as maintenance hosts, such as *Passer domesticus*

which are implicated as an ecologically significant host in the USA (Reisen et al., 2000), are present in the UK. There are both known vector species and known reservoir hosts in the UK and therefore virus introduction presents some risk of ongoing transmission.

VEEV

VECTORS

Culex melanoconion species are considered the natural vectors of enzootic strains and epizootics involve adaptation to other genera such as *Psorophora* and *Aedes (Ochlerotatus*) and other *Culex* species (Pages et al., 2009). At least 41 species belonging to 11 genera of mosquitoes have been reported to have been found naturally infected with VEEV strains; all were exclusively American species. Non-mosquito arthropods have also been implicated in mechanical transmission of VEEV (Weaver et al., 2004).

VECTOR SPECIES PRESENT IN EUROPE

No European mosquito species have been competence tested for VEEV. Endemic vector species are of the subgenus *Melanoconion* (Ferro et al., 2003; Galindo, 1971), which are not present in Europe. Epidemic virus has a broad vector range. Therefore, European mosquitoes may be able to vector these strains. All studies on vector competence have been carried out on exclusively American species with the exception of *Culex pipiens*, which is non competent (Turell, 2012) and the invasive species *Aedes albopictus* (Fernandez et al., 2003; Smith et al., 2005), which is a competent laboratory vector for some epidemic strains but has never been implicated in field transmission.

BRITISH CANDIDATE VECTORS

Horses are the major amplification host of epidemic VEEV, which has a broad vector range. Therefore, potential bridge vectors (*Aedes vexans, Ochlerotatus spp., Culiseta spp.*) are candidates for laboratory competence testing.

ONGOING TRANSMISSION POTENTIAL

Known significant reservoir host species of enzootic VEEV are not present, so it is much less likely that enzootic transmission would occur in the UK, compared to epizootic transmission.

RRV

VECTORS

A number of different mosquito species are involved in various areas and seasons for example *Aedes camptorhyncus* and *Aedes vigilax* (northern and southern saltmarsh mosquitoes) are significant ecological vectors and *Cx. annulirostris* is important in inland areas. Various other *Aedes* species are involved depending on the conditions and region. RRV has been recorded in at least 42 species in Australia of different genera including *Anopheles, Coquillettidia, Culex, Culiseta and Aedes* including *Ochlerotatus* and at least 10 species have been shown to transmit virus in laboratory studies (Russell, 2002). RRV causes outbreaks in varied environments in Australia including both temperate and tropical areas (Russell, 2002).

VECTOR SPECIES PRESENT IN EUROPE

Common vectors of RRV in Australia are not present in Europe, however the invasive mosquito *Aedes albopictus*, which is present in Europe, is able to transmit RRV (Rosen et al., 1981).

BRITISH CANDIDATE VECTORS

As RRV has a broad host range, any mammalophagic mosquito could be a candidate vector. Therefore, *Ochlerotatus spp*, and *Culiseta spp.* should be investigated for laboratory competence.

ONGOING TRANSMISSION POTENTIAL

Recent evidence suggests that macropods may not be required for ongoing transmission, as was previously thought (Lau et al., 2017) and that human beings may act as reservoir hosts. It is therefore possible that if suitable vectors exist in the UK, ongoing transmission could be possible for RRV, particularly as this virus is known to replicate efficiently in temperate climates (Kay and Jennings, 2002; Russell, 2002).

GETAH VIRUS

VECTORS

Aedes vexans niponii and *Culex* spp. have been implicated as the major vectors in endemic areas (Fukunaga et al., 2000).

VECTOR SPECIES PRESENT IN THE UK

The known major vectors are not present in the UK.

BRITISH CANDIDATE VECTORS

Getah virus has been isolated in *Aedes euedes* and other unidentified *Aedes spp*. in Siberia in the subarctic zone (Mitchell et al., 1993), suggesting that it is able to adapt to vector species present in a wide range of climatic zones. Therefore, *Aedes* and possibly *Ochlerotatus* spp. are potential candidate vectors in the UK and could be investigated for laboratory vector competence.

ONGOING TRANSMISSION POTENTIAL

Amplification hosts (swine, horses) are present in the UK. It is not known which species act as enzootic reservoirs, so these species may, or may not, be present in the UK.

1.3.4 Mosquito-horse interaction

There is little information available on the host-vector interaction between mosquitoes and horses in the UK, and mosquitoes have not been specifically sampled on equine premises. Therefore, it is not possible to say what level of exposure UK horses have to biting by known or candidate vector species. What information we do have concerns only evidence that a particular mosquito species is known to have blood-fed from an equid, rather than an idea of the frequency of this behaviour. Species present in the UK which have been demonstrated to blood-feed from horses (globally) include *Ae. cinereus, Ae. vexans, An. claviger, An. maculipennis s.l., An. plumbeus, Cq. richiardii, Cs. annulata, Cs. subochrea, Cx. modestus, Cx. pipiens s.l., Oc. annulipes, Oc. cantans, Oc. caspius, Oc. detritus, Oc. dorsalis, Oc. flavescens, Oc. geniculatus, Oc. punctor, Oc. rusticus and Ochlerotatus sticticus* (reviewed in Chapman et al., 2016, Chapter 2). A study of 6 equine premises conducted in Belgium sampled several of the species present in the UK: *An. claviger, An. maculipennis s.l., An. plumbeus, Cq. richiardii, Cs. annulata, Cx. pipiens s.l. Cx. torrentium, Oc. cantans,* and *Oc. geniculatus* (Boukraa et al., 2016). Of these species *An. maculipennis s.l., An. plumbeus, Cq. richiardii, Cs. annulata*, and *Cx. pipiens s.l.* are vectors or laboratory vectors of equine arboviruses (Chapman et al., 2016, Chapter 2). The container breeders *Cx. pipiens s.l., Cx torrentium* and *Cs. annulata* were found on all 6 sites (Boukraa et al., 2016). As several of the species sampled in this study are common and widespread in the UK it seems likely that British horses are regularly exposed to mosquitoes, including vector species. Studies in France (on 2 equine premises) and Switzerland have shown, using host-baited traps, that several species present in the UK will bite horses frequently: *An. claviger, An. maculipennis s.l., Ae. vexans, Cs. annulata, Cq richiardii, Cx. modestus, Oc. cantans, Oc. caspius, Oc. detritus* (Balenghien et al., 2006; Schönenberger et al., 2016). Of these, only *An. claviger* and *Oc. cantans* have not been

demonstrated to be vectors or laboratory vectors of equine arboviruses (Chapman et al., 2016, Chapter 2).

To assess the potential for host-vector interaction in the event of a future outbreak of equine arboviral, disease in the UK and to aid in the design of surveillance strategies, baseline data on the species composition and abundance of mosquitoes which may interact readily with equines is important. This information is currently lacking.

Arbovirus Disease Prevention and Control

1.4.1 Surveillance

In the UK there is currently no active surveillance for arboviruses affecting horses (unless human donor blood supply levels require testing rather than deferment of donations after visiting an WNV affected area). Autochthonous WNV infection in humans should be reported to National Surveillance Centres, but in more than a third of cases of encephalitis the causative organism is not identified (Gossner et al., 2017). West Nile fever is a notifiable disease of equidae in the UK, and monitoring relies on passive surveillance of horses with neurological signs. Passive surveillance of birds is in place between April and October (Gossner et al., 2017). There is no specific surveillance plan for other mosquito-borne arboviruses of horses, and they are not notifiable.

Modelling based on summer temperatures can be used to predict transmission risk in particular regions at the end of that transmission season, when most clinical cases are likely to be seen (Paz et al., 2013). These predictions can be used for early warning of risk to the UK. In this scenario perhaps the most effective ways to increase surveillance are to use sentinel birds and test mosquitoes for virus RNA, both allowing identification of WNV circulation before human clinical cases occur (Chaintoutis et al., 2014; Healy et al., 2015). However, both strategies are expensive. Serosurveys of horses could be used retrospectively if an outbreak were suspected in the UK, to investigate its extent. In countries where regular outbreaks occur, equine surveillance is not favoured due to increases in vaccine uptake, and the fact that equid infections may not pre-date human infections (Dauphin et al., 2004). With regards to WNV, surveillance activities in other countries, such as France, are likely to give an early warning of increased risk to the UK.

However, for viruses which have never been transmitted in Europe this may not be the case, as direct importation into the UK, or importation from Europe prior to outbreak recognition could occur. Mathematical and epidemiological modelling can help to identify

subpopulations, regions and periods when there is a higher risk of entry and spread of specific diseases, enabling the occurrence of outbreaks to be anticipated through targeted surveillance. For example, a study to identify hotspots for potential introduction of EEEV, WEEV, VEEV and JEV to Europe through live animal trade demonstrated that the risk was higher for EEEV than the other three viruses, which was mainly associated with trade in exotic pet species such as rodents, reptiles or caged birds (Durand et al., 2013). The risk was greatest in Belgium, the Netherlands and northern Italy, highlighting that managing the risk of introduction of exotic arboviruses to Europe as a result of animal trade requires a transboundary and international approach. The UK may (at least in the near future) be more at risk from those viruses which are known or suspected to be transmitted at lower temperatures or in more temperate regions such as WNV, RRV, Getah virus and WEEV.

HORSE OWNER AWARENESS OF EQUINE ARBOVIRAL DISEASE

As passive surveillance is used in the UK for arboviruses causing disease of horses (currently only WNV and African horse sickness are notifiable) it would be beneficial if horse owners as well as veterinarians were aware of the clinical signs of these diseases (Sabirovic et al., 2008a), to increase the likelihood that suspicious disease will be reported, and appropriate testing undertaken. However, information regarding the knowledge of horse owners in the UK about both endemic and non-endemic infectious disease is not available. Survey information is available regarding vaccination or non-vaccination for influenza, but details of disease knowledge have not been investigated (Boden et al., 2013; Hotchkiss et al., 2007; Mellor et al., 2001). In fact, there is little available information in a global context regarding horse owner knowledge of equine infectious disease, although studies in Australia have been carried out subsequent to the influenza outbreak in 2007 (Schemann et al., 2012) and the emergence of Hendra (Kung et al., 2013; Manyweathers et al., 2017; Taylor et al., 2016). Studies on Hendra reveal cause for concern over horse owner understanding of the risk associated with this zoonotic disease, and their distrust in the motivation of veterinarians (Goyen et al., 2017). If future surveillance in the UK is to rely upon identifying clinical cases in horses and humans, then information regarding the awareness of signs of disease in both the veterinary profession and equine sector is imperative. This will allow policymakers to assess the importance of educational campaigns in the event of heightened disease risk.

1.4.2 Protection of Horses

Control measures for prevention of disease dissemination, involving movement restrictions or other controls upon horses, are only relevant for epidemic VEEV and RRV, although for RRV movement restrictions on horses may be less successful due to the ability of humans to

infect vectors. Limiting the spread of the other equine arboviruses is much more challenging, even in non-endemic countries, due to the complex transmission cycles and wildlife hosts.

VACCINATION

Successful equine vaccines are available for most of the common equine arboviruses with the notable exception of RRV and MVEV. An equine licensed WNV vaccine is available for use in the UK, for competition horses travelling to areas of WNV activity. Multivalent inactivated vaccines including WNV, EEEV, WEEV and VEEV are used in the USA. WNV vaccines have included live canarypox virus vectored vaccine, inactivated vaccines and DNA vaccines. Traditionally, inactivated vaccines were derived from mouse brain; however cell culture derived vaccines are now also used (Ishikawa et al., 2014).

There are no commercially available vaccines for humans for seven of the eight arboviruses discussed here, with the exception being JEV, despite successful clinical trials in some cases (Wressnigg et al., 2015). However this seems to be in part due to cost benefit analysis and commercial considerations regarding the cost of producing these vaccines, against the low incidence of clinical disease globally (Ishikawa et al., 2014).

In the event of increased risk to the UK from arboviruses other than WNV, it would be important to encourage drug companies to apply for vaccine licences. Investigation of the attitude to horse owners regarding multiple vaccination schedules would be useful, in assessing what information and advice veterinarians should provide, if further vaccinations were licensed in the UK. Many owners in the UK will only be familiar with tetanus and influenza vaccination (Hotchkiss et al., 2007).

PROTECTION OF HORSES FROM MOSQUITO BITING

In the event of increased risk of disease, many horse owners will want to provide their horse(s) with protection from infection, and if vaccines are not yet available, then other methods of mitigating risk will be particularly important. Some owners may choose not to vaccinate and prefer to mitigate risk by reducing mosquito biting of their horse. Methods to reduce mosquito biting (available in the UK) include topical insecticides, insect-proof barriers for stabling, repellents and fly-rugs including those impregnated with permethrin. Control of mosquito populations by removing breeding sites such as stagnant water or treating potential sites with the ‘biological pesticide’ *Bacillus thuringiensis israelensis* is also possible. Significant proportions of horses in the UK are kept at pasture for most of the day during summer and are not kept on premises belonging to their owner (Hotchkiss et al., 2007). This means that individual, horse-level measures of protection may be preferred and faster to implement. These methods include repellents and the barrier protection (fly rugs

and masks). There are no peer-reviewed, published field studies specifically investigating the efficacy of these methods for the protection of horses from mosquito biting. Therefore evidence-based advice is currently not available to stakeholders.

Horse Owner Perception of Bite Protection Methods

Little peer-reviewed information is available regarding the products or methods used by UK horse owners for protection of horses from fly biting and that which is available is not up to date with currently available products (Biggin et al., 1999). This is despite the fact that *Culicoides* hypersensitivity is common in horses in the UK (McCaig, 1973). In a survey of horse owners conducted by a UK equestrian magazine 28% of participants used a product containing citronella oil, 99% of these used it for repelling flies (Horse & Hound, 2015). This is concerning because citronella oil is not considered effective as a repellent and cannot be marketed as such, for use on animals, in the European Union (European Chemicals Agency, 2017). Further information about current use of fly-bite protection methods used by horse owners would be useful to the veterinary profession with regards to giving advice for all applications of these methods, including both allergic and infectious disease mitigation.

Summary

Equine arboviruses cause significant welfare and economic burdens on the equine industry in endemic countries. The epidemiology, transmission cycles and vector ecology relating to the mosquito-borne arboviruses affecting horses are incredibly complex and in some cases poorly understood, even in those countries. Knowledge of potential wildlife reservoirs and candidate vectors which are not present in endemic countries is understandably poor. It is therefore beyond the scope of this thesis to consider overall risk of introduction or probability of ongoing transmission and spread of these viruses in the UK. However, it is conceivable that either WNV or one of the other arboviruses may pose a risk to British horses in future. In this Chapter, known vector species present in the UK and candidate British vector species have been described, and further investigation of candidate vector ecology, including host-vector interaction and vector competence is imperative if further knowledge of the risk of arbovirus transmission is to be gained. In particular vector competence, or lack thereof, of British mosquito populations is critical information for predicting and assessing the consequences of a virus introduction event and requires further investigation. In the event of a suspected outbreak of equine arboviral disease in the UK, or predicted high risk of an outbreak, vaccines may not be immediately available and therefore

the efficacy of other protection methods is important information for policy makers and the equine sector. Horse owners are key stakeholders in the event of infectious disease outbreaks. Therefore, to minimise welfare and economic impacts in the event of an outbreak of disease, further information is needed regarding their knowledge of, and attitudes to aspects of these diseases and their management.

Aims

The work presented in this thesis focusses on the risk of autochthonous (in-country) transmission in the UK after virus introduction, specifically: the potential for host-vector interaction, vector competence of British populations of mosquitoes, the efficacy of protection methods for individual horses and horse owner knowledge of disease and prevention:

- Chapter 2 investigates the presence of mosquito species on equine premises across England
- Chapter 3 investigates the knowledge of, and attitudes towards, biting insects and arboviral disease of horses, amongst UK horse owners
- Chapter 4 demonstrates the vector competence of several Palearctic (British) species of mosquito for arboviruses affecting both equines and humans
- Chapter 5 demonstrates the level of protection afforded by two products, commonly used as fly repellents by horse owners, against UK mosquitoes in the field
- Chapter 6 investigates performance of six spray repellent products available on the equine market in the UK, against the Palearctic saltmarsh mosquito *Oc. detritus* in the laboratory
- Chapter 7 places in context the findings of studies in this thesis, with regards to the future risk to UK horses from mosquito-borne arboviruses

2 MOSQUITO SPECIES PRESENCE ON EQUINE PREMISES IN THE UK

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Potential vectors of equine arboviruses in the UK

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Abstract

There are several mosquito-borne viral diseases that cause varying levels of morbidity and mortality in horses and that can have substantial welfare and economic ramifications. While no equine arboviral diseases have yet occurred in the UK, vector species for some of these viruses are present, suggesting that UK equines may be at risk. However detailed information about mosquito vector distribution and therefore, the opportunity for horse-vector interaction is lacking. In this chapter, the first study of mosquito species on equine premises in the UK is presented. Regions in the North West, North East, South East and South West of England were sampled. Mosquito Magnet traps and red-box traps were used to sample adults, and larvae were collected from water sources such as tyres, buckets, ditches and pools. Several species which are known to be capable of transmitting important equine infectious arboviruses were trapped. The most abundant, with a maximum catch of 173 in 72 hours was *Ochlerotatus detritus*, a competent vector of some flaviviruses; the highest densities were found near saltmarsh habitats. The most widespread species, recorded at >75% of sites, was *Culiseta annulata.* This study demonstrates that potential mosquito vectors of arboviruses, including those known to be capable of infecting horses, are present and may be abundant on equine premises in different regions of the UK.

Introduction

Despite the worldwide significance of arboviral disease in equine populations (Table 1.3) and the status of the horse as a sentinel species for early warning of the likelihood of human disease outbreaks (Faverjon, 2015) the relationship between the horse and mosquito vectors has not been specifically studied in the UK.

Further knowledge about potential vector mosquitoes in the UK and their ability to spread arboviruses will play a key role in control and surveillance of disease in the event of an outbreak. There are 34 species of mosquito in the UK (Medlock and Vaux, 2011) and species that are implicated as vectors of arboviruses of horses elsewhere in the world include *Aedes cinereus, Ae. vexans, Anopheles maculipennis s.l., Coquilletidia richiardii, Culex pipiens, Cx. modestus, Culiseta morsitans, Ochlerotatus caspius, Oc. dorsalis and Oc. flavescens* (Table 2.1). A number of these are widely distributed and locally abundant across the UK (Table 1.6). In addition, some mosquito species present in the UK have been shown in the laboratory to be competent vectors of at least one of these viruses including *Oc. punctor* (WNV), *Oc. detritus* (WNV, JEV), *Cx. modestus* (WNV), *Ae. vexans* (WNV, EEEV), *Cx. pipiens s.l* (WNV, JEV), and *An. plumbeus* (WNV) (Table 2.1).

Species	Host Biting[5,7]	Evidence of Equine Biting	Vector Status
Ae. cinereus / geminus	M[31, 32] B[31, 32]	Morocco[1] Switzerland[32]	EEEV [I][18]
Ae. vexans	M[31, 32] B[31,32]	France[2] Switzerland[32]	WNV [I][5] EEEV [IL"][18,19,20,21,22,]
Anopheles algeriensis	M		
An. claviger	M[32]	Switzerland[32]	
An. maculipennis s.l.	M[31,32] B[31]	UK[4,8] Switzerland[32]	WNV [I][5]
An. plumbeus	M[31]	France[2]	WNV [L][14]
Cq. richiardii	M[31,32] B[32]	France[2] Switzerland[32]	WNV [I][5]
Culiseta alaskaensis	M		
Cs. annulata	M[14,32] B[32]	UK[3], France[2] Switzerland[32]	WNV [L][16]
Culiseta fumipennis	B		
Cs. litorea	M B		
Culiseta longiareolata	B		
Cs. morsitans	M[31] B[31]		EEEV [Z][17, 19]
Cs. subochrea	M[2]	France[2]	
Culex europaeus	A R B		
Cx. modestus	M[2] B[2]	France[2]	WNV [V L][2.5]
Cx. pipiens s.l.	M[31] B[31,32]	France[2]	WNV [V L][23, 27] JEV [L*][23] EEEV[N][26] WEEV [N][24,25] VEEV [N][27]
Cx. torrentium	B[31] M[31]		
Oc. annulipes	M[9,31] B	France[2]	
Oc. cantans	M[31] B[32]	UK[9] Switzerland[32]	
Oc. caspius	M[2] B[2]	UK[3], France[2]	WNV [I L*][2,5]
Ochlerotatus communis	M[31]		
Oc. detritus	M[2] B	UK[3], France[2]	WNV [L][16] JEV [L][16]
Oc. dorsalis	M[6]	UK[6]	WEEV [I L][28,30]
Oc. flavescens	M[11,12]	Denmark, Canada[11, 12]	
Oc. geniculatus	M[2,31]	France[2]	
Ochlerotatus leucomelas			
Oc. punctor	M[10] B	UK[10]	WNV [L][14]
Oc. rusticus	M[31, 32] B	Switzerland[32]	
Oc. sticticus	M[31, 32] B[31]	Switzerland[32]	
Orthopodomyia pulcripalpis	B		

Table 2.1 Mosquito species present in the UK, horse and mammal biting, and vector status for arboviruses of horses.

Species underlined were sampled during the present study.
A- amphibians, B – birds, M – mammals, R – reptiles, L – Laboratory competent vector,
I – Implicated in disease transmission worldwide, N – Non-competent as laboratory vector,
V – Ecologically sign significant bridge vector worldwide, Z – Ecologically significant enzootic vector worldwide,
* -Relatively inefficient laboratory vector, " – Variable laboratory competence in a number of studies.

1. (Faraj et al., 2009)
2. (Balenghien et al., 2006)
3. Work for this thesis
4. (Danabalan, 2010)
5. (Medlock et al., 2005)
6. (Service, 1971b)
7. (Becker et al., 2010)
8. (Hutchinson, 2004)
9. (Medlock and Vaux, 2011)
10. (Service et al., 1986)
11. (Service and Smith, 1972)
12. (Rempel et al., 1946)
13. (MacKenzie-Impoinvil et al., 2014)
14. (Vermeil et al., 1960)
15. (Balenghien et al., 2008)
16. Marcus Blagrove, unpublished observation
17. (Andreadis et al., 1998)
18. (Armstrong and Andreadis, 2010)

19. (Centers for Disease Control and Prevention (CDC), 2006)
20. (Vaidyanathan et al., 1997)
21. (Davis, 1940)
22. (Chamberlain et al., 1954)
23. (Turell et al., 2006)
24. (Aviles et al., 1990)
25. (Hammon and Reeves, 1943)
26. (Merrill et al., 1934a)
27. (Turell, 2012)
28. (Kramer et al., 1998)
29. (Vaux et al., 2015)
30. (Zacks and Paessler, 2010)
31. (Börstler et al., 2016)
32. (Schönenberger et al., 2016)

In recent years Public Health England has been involved in both passive and active surveillance of mosquito populations in the UK (reviewed briefly in Engler et al., 2013). This work has encompassed port and transport network, and tyre import surveillance for the incursion of invasive species, population impacts of wetland scheme expansion on endemic species, and general surveillance on 10 nature reserves across England (Medlock and Vaux, 2011, 2013, 2014; Vaux et al., 2011; Vaux and Medlock, 2015). Active surveillance methods used by Public Health England included the use of Mosquito Magnet traps, larval sampling, oviposition traps and BG-sentinels. These surveillance efforts are considered important in the context of future risk to the UK, to aid in assessment of that risk, and in preparedness. Invasive species, if established could substantially increase the risk of transmission of arboviruses in the UK, not to mention the risk of virus introduction with these vectors. The potential future risk of virus transmission or nuisance biting associated with wetland expansion, and increased local mosquito populations, is also important to assess, so that policy-makers can be advised of the advantages and disadvantages of policies encouraging such landscape change (Medlock and Vaux, 2011). General surveillance of native mosquito populations in wetland nature reserves allows for pathogen analysis across England (Engler et al., 2013), and allows for monitoring of populations over time. This may allow for prediction of significant increases in host-vector interaction due to mosquito population increase. Recent interest in UK mosquitoes and increased sampling has led to the discovery of the West Nile virus vector *Cx.modestus* (Cull et al., 2016; Golding et al., 2012) and eggs of the invasive mosquito *Ae. albopictus* in Kent (Medlock et al., 2017), and attempts to map expected distribution of potential WNV vector species (those which are implicated in transmission in continental Europe) in the UK (Golding, 2013). Increased surveillance provides some baseline knowledge to allow for future modelling of species distribution expansions with climate change. Absence data is however very difficult to produce for species with a patchy distribution (Thompson, 2012a). This is particularly pertinent to mosquitoes in the UK, which often have a localised distribution, have not been studied closely as vectors in the past, and are often difficult to sample. This makes predicted distributions based on climate and geographical data important, allowing a combination of presence data and modelling to inform risk assessment for host-vector interactions.

Studies of mosquito populations associated with equine premises have been conducted in Europe (Balenghien et al., 2006; Boukraa et al., 2016), however, in the UK there has been no sampling of mosquito species with a specific focus on the equine host. Accordingly, we carried out a survey of the mosquitoes present at 32 premises across England to obtain baseline data on the species composition and abundance of mosquitoes that may interact readily with equines. Our results identify which species may play an important role in outbreaks of mosquito-borne equine viruses in the UK and hence contribute to the development of national strategies to monitor and manage this risk.

Methods

2.3.1 Consideration of trapping methods

HOST-SEEKING FEMALES

There are a large number of trapping methods which have been used for the sampling of mosquitoes worldwide (Silver and Service, 2008). Some of the most commonly used methods in adult mosquito surveillance worldwide are the CDC light trap (various models), Encephalitis Vector Survey traps, Biogents (BG) Sentinel trap and the Mosquito Magnet trap. Some of these may be used with or without carbon dioxide such as the BG-sentinel.

In Europe, in recent years, carbon dioxide-baited traps including the Mosquito Magnet and BG-Sentinel have been favoured for surveillance due to their success, and convenience in not requiring daily battery replacement, or dry-ice (Engler et al., 2013; Golding et al., 2012; Vaux et al., 2011). In the UK, it has been reported that mosquito sampling without the use of carbon dioxide as bait produces limited results and the Mosquito Magnet is accepted as the best way to trap larger numbers of mosquitoes of most mammal-biting species than other methods. In the past reliability of Mosquito Magnet traps has been reported as a problem (Hutchinson et al., 2007), but that study used an older model of the trap, and the current model may be more reliable. BG-sentinel traps are easier to site than CDC miniature traps as they can be placed on the ground as well as being hung in suitable sites. CDC traps may be less suitable for UK use, due to unpredictable precipitation making sample handling difficult and leading to loss of scale patterns which are necessary for identification. Although the Mosquito Magnet will damage samples to some extent (due to the powered fan) they are protected from rain.

The BG-sentinel trap or CDC miniature trap were considered an option for local surveillance as small amounts of dry ice, which act as a source of carbon dioxide, could be potentially replenished daily. However logistically the safe transport of enough carbon dioxide cylinders or dry-ice to bait traps in a study with a wide geographical range, such as that described here, was considered impractical: propane canisters for the Mosquito Magnets could be delivered to each site by the supplier. Therefore, the Mosquito Magnet was chosen for trapping of host-seeking mosquitoes in this study.

BLOOD-FED MOSQUITOES

There are limited reports of methods used in the UK to collect blood-fed mosquitoes, although in some instances collection from buildings (by aspiration) or vegetation (by sweep netting)(Brugman, 2016; Danabalan, 2010; Hutchinson, 2004; Service, 1971b) have been successful, but the collection of these mosquitoes in the field is difficult (Brugman et al., 2015), with specific local knowledge of mosquito resting places being a distinct advantage. However, resting box traps have been used with some success in specific sites in the UK (Brugman, 2016). Pilot work in September 2014 at site 8 (Table 1) included two 15-minute sampling efforts, sampling mosquitoes whilst they were taking blood meals from horses. This yielded 20 *Oc. detritus,* 3 *Oc. caspius,* and 2 *Cs. annulata* and therefore sampling directly from horses (host-landing catch) was considered a potentially useful adjunctive method for trapping blood-fed mosquitoes to be used in this study.

Traditionally much of the information regarding blood meal sources of British mosquitoes has been obtained with the use of precipitin testing or enzyme-linked immunosorbent assay (Service, 1971b; Service et al., 1986). Although these techniques are seldom used now, they are still considered perfectly adequate for differentiation between orders of animals (Kent, 2009) and therefore information regarding equid blood meals in the UK can be relied upon. More recently molecular methods based on amplification of cytochrome B oxidase gene (Danabalan, 2010) or the cytochrome oxidase I gene (Brugman, 2016) of the host, have been employed, providing evidence of equine biting for several mosquito species in UK populations (Table 1.6).

2.3.2 Region and Site Selection

The ecological sampling of rare species and those with a patchy distribution commonly utilises stratified sampling methods (Thompson, 2012b). In this case, four types of mosquito breeding habitat were identified: land associated with drainage ditches (drained farmland) or fenland, woodland, urban (urban and suburban habitat classes in UK Land Cover map

(Burns, 2014)) and saltmarsh (Hutchinson et al., 2007). Two sites in each category, per region, was the aim as this was considered an absolute minimum sample size. More sites could not be utilised due to logistical and financial constraints. Therefore, the choice of study regions had to consider the availability of these types of habitats, as significant areas of fenland or wetland and saltmarsh habitats are not found in all geographical regions. Secondly, regions were excluded if the density of identifiable equine premises did not allow for practical logistics.

2.3.3 Choice of Sites

A total of 32 sites were sampled - 8 equine premises in each of North West, North East, South East and South West regions in England. Four types of mosquito breeding habitat were identified: land associated with drainage ditches (drained farmland) or fenland (site 29), woodland, urban and saltmarsh (Hutchinson et al., 2007). We aimed to recruit two equine premises in each of the four habitats in each region (32 premises in total).

An internet search was conducted using Google Maps and The Phone Book from British Telecom, using the search terms 'Riding Schools', 'Livery', 'Stables', 'Stud'. BHS Riding Schools and Livery Yard Lists and the British Equestrian Directory and Newmarket Trainers Association lists were also utilised**.** This produced a list of businesses with publicly available contact details.

For each premises the local area was investigated for potential mosquito habitats using Magic (www.magic.gov.uk) and Google Earth. Sites were assigned a category based on habitat (some sites qualified for two categories) and were graded based on the area of presumed habitat and proximity of habitat to the premises. We aimed to locate premises within suitable habitats or, if that was not possible, within 500 m (woodland and urban sites), 1 km (urban and drained farmland sites) or 3 km (saltmarsh). A maximum distance of 500 m for woodland sites was selected reflecting the relative ease of finding sites close to woodland. For saltmarsh or grazing marsh it was not possible to find sites in close proximity in many cases, but species associated with floodwaters, such as *Aedes vexans* and coastal saltmarsh such as *Oc. detritus*, tend to have greater dispersal capacity and *Oc. detritus* is capable of flying at least 2.5 miles (Becker et al., 2010; Service, 1969, 1971c; Snow and Medlock, 2008; Verdonschot and Besse-Lototskaya, 2014). In order to try and include all four habitat types within reasonable travelling distance, the four areas within the regions were chosen as follows: Wirral peninsula and Chester (North West); between Scunthorpe, Gainsborough, Doncaster and Goole (North East); within 20 miles of Exeter (South West); a transect from Newmarket to the Wash (South East).

Premises were recruited by sending out either a letter or e-mail to the business selected and following this up with a telephone call. For sites where there was no response or a negative response, correspondence was then sent to a number of alternative second choice sites, for that category of habitat, until 32 sites (8 in each of four regions) were recruited (Figure 2.3).

2.3.4 Fieldwork Protocols

Ethical approval for this study was obtained from the University of Liverpool Veterinary Ethics Committee (VREC258).

Each of the 32 sites was visited three times throughout the summer of 2015, and mosquitoes were trapped continuously for three days. Timing of visits was based on what is presently known about peaks in adult mosquito numbers of different species in the UK, visiting each of four regions within each of 3 seasonal peaks of mosquito activity in the months of May, late June-early July and September (Becker et al., 2010; Medlock et al., 2007; Medlock and Vaux, 2015; Service, 1969, 1977; Snow and Medlock, 2008).

SAMPLING METHODS

HOST SEEKING ADULTS

Trapping on each site consisted of a Mosquito Magnet, Independence model (Woodstream Europe Ltd; Figure 2.1) and a resting box trap. The Mosquito Magnet is designed to catch host-seeking mosquitoes by using propane as a fuel source to produce heat, moisture and carbon dioxide. The trap was additionally baited with 1-octen-3-ol (as supplied by the trap manufacturer). The Mosquito Magnet trap was run continuously for ~ 72 hours starting in the morning and a data logger was placed underneath the body of the trap to record the environmental temperature and relative humidity for this time-period.

Figure 2.1 Mosquito Magnet Trap.

Attempts were made to catch mosquitoes landing on hosts to confirm that mosquitoes were taking blood meals from horses. Four sites in each geographic area were sampled in June/July and September in the mid-late afternoon, and four sites around dusk. For each sampling effort, a group of horses was observed for fifteen minutes, to see if any mosquitoes could be identified landing on them. If no mosquitoes were observed, then another group of horses was observed for 15 minutes. Group sizes ranged from 1 to 10, as horses were in their normal grazing environment (with the exception of site 25 where sampling was attempted in the stable as there was no grazing). If no mosquitoes were observed on two groups the attempt was abandoned. If mosquitoes were observed, landings were counted for 2 minutes and then mosquitoes were sampled from the head and neck of the horse (for reasons of safety) for 30 minutes, to allow for species identification. Some premises could not be sampled at dusk due to access restrictions, so were only sampled in the afternoon. In order to trap mosquitoes feeding on horses, a mechanical pooter (Watkins and Doncaster) was modified with an elongated inlet tube and was muffled, so as to avoid startling the horse. Individual horse behaviour was discussed with the yard owner in advance (to confirm that the horse was not expected to be distressed, or display aggressive behaviour), and permission to attempt landing catches with each horse or group of horses was obtained.

RESTING ADULTS

The capture of resting mosquitoes was attempted using knock-down catches, sweep netting and using a resting box trap: a 40x30x20cm black box (Brugman, 2016; Morris, 1981), painted red inside, designed to aid in the capture of blood-fed mosquitoes. It was set in an open area facing west and was emptied on two mornings, (either at 24 and 72 hours after deployment, or 48 and 72 hours) by placing a perspex cover on the open front of the box and aspirating resting mosquitoes.

Figure 2.2 Red box trap (Chapman et al., 2016).

IMMATURE MOSQUITOES

Larval sampling was undertaken on the equine premises themselves and, where there was access, on neighbouring land within 500 m of the Mosquito Magnet or of grazing horses. The aim was to sample all water sources within the boundary of the premises, including all collections of artificial containers. This was not always possible due to access constraints or on larger premises. Larvae and pupae were sampled using a dipper. This is a 500 ml cup shaped ladle with a long handle. Each dip was then emptied into a white tray and searched for larvae. For larger waterbodies 5 x 500 ml dips were used in different parts of the water-body. This was not randomised because often, sampling was restricted to areas convenient to access, and presence rather than abundance was investigated. For small containers only one dip sample or partial dip samples could be obtained.

MOSQUITO STORAGE

Mosquitoes were removed from the Mosquito Magnet net with a mechanical aspirator and ‘Fly-nap’ (Carolina Biological Supply Company, Burlington, NC, USA) was used to

produce knock-down. Adult mosquitoes were stored dry, were morphologically identified within 4 days and were then stored in 90% ethanol. Blood-fed mosquitoes were stored in 90% ethanol immediately.

Larvae were pipetted into 30ml universal containers (Starlab, Milton Keynes, UK) for storage. Fourth instar larvae were killed by gradually adding 90% ethanol. Pupae were allowed to emerge for ease of identification. Live 2nd and 3rd instar larvae were allowed to continue to develop until the end of the fieldwork week for ease of identification. Containers were inspected daily and any dead larvae or pupae were preserved using 90% ethanol for identification (Snow, 1991).

2.3.5 Mosquito Identification

Mosquitoes of all stages were identified morphologically as far as possible, to species or species complex using keys of British and European mosquitoes (Becker et al., 2010; Cranston et al., 1987; Marshall, 1938; Schaffner et al., 2001; Snow, 1991). *Cx. pipiens* was differentiated from *Cx. torrentium* by molecular methods as described by Hesson and others (2010), detailed in the next section.

MOLECULAR DIFFERENTIATION OF *CX. PIPIENS* AND *CX. TORRENTIUM*

DNA extraction from mosquitoes was achieved using the E.Z.N.A. MicroElute Genomic DNA Kit (Omega Bio-Tek, Inc., Norcross, GA, U.S.A.):

Mosquitoes were removed from the storage vials and were placed on absorbent paper to allow the ethanol to evaporate. Individual larvae or adults were placed in a 1.5 ml Eppendorf tube, 200 µl TL Buffer and 20 µl OB Protease Solution were added and mosquitoes were homogenised using a micropestle. After incubating at 55 °C for 4 hours (or overnight) the tubes were centrifuged (13,000 x g) for 2 minutes. The supernatant was transferred to a clean Eppendorf tube and 220 µl of BL buffer was added. Tubes were vortexed briefly and were then incubated at 70 °C for 10 minutes. 220 µl of ethanol was then added and tubes were vortexed at maximum speed for 15 seconds, and briefly centrifuged. The sample was transferred to a DNA Mini Column and 500 µl of HBC buffer was added. The column was centrifuged (13,000 x g) for 30 seconds and the filtrate discarded. 700 µl DNA Wash Buffer was added to the Mini Column and it was centrifuged (13,000 x g) for 1 minute. The Wash Buffer step was repeated. The empty Mini Column was centrifuged (13,000 x g) for 2 minutes. The Mini Column was transferred to a 1.5 ml Eppendorf and 50µl Elution Buffer (at 70°C) was added. The Mini Column was incubated at room temperature for 3 minutes

and centrifuged (13,000 x g) for 1 minute. The Mini Column was removed, and eluted DNA stored at -20°C.

Primers, PCR amplifications and restriction enzyme digestion were previously described (Hesson et al., 2010). The primer pair used to amplify an approximately 830 bp fragment of the COI-3' region of mitochondrial DNA were C1-J-2183, sequence CAACATTTATTTTGATTTTTTGG and TL2-N-3014, sequence TCCAATGCACTAATCTGCCATATTA. PCR amplifications were carried out in 20 µl reactions with final concentrations of 1x PCR buffer, 2.5 mM $MgCl_2$, 0.125 mM of each dNTP, 0.01% DMSO, 0.4 µM of each primer, 0.25 unit of Taq DNA polymerase (Thermo Fisher Scientific, Inc., Waltham, MA, USA), and 2µl of purified DNA. The PCR reaction was performed under the following conditions: an initial denaturation step of 95 °C for 3 minutes, followed by 5 cycles of 94 °C for 30 seconds, 47.8 °C for 30 seconds, and 72 °C for 1 minute, and then 30 cycles of 94 °C for 30 seconds, 49.8 °C for 30 seconds and 72 °C for 60 seconds, and ending with a final extension at 72 °C for 7 minutes.

The restriction enzymes FspBI (BfaI) and SspI were used to produce a diagnostic banding pattern on agarose gel to differentiate the two species. The enzyme FspBI recognises C'TAG present in *Cx. torrentium* and produces two fragments (~210b p and ~620 bp) whilst the PCR product of *Cx. pipiens* remains uncut. The enzyme SspI recognises AAT'ATT and PCR products of *Cx. pipiens* are cut into two fragments (~210 bp and ~620 bp), whilst *Cx. torrentium* product remains uncut.

Restriction enzyme digestion reactions were carried out in a reaction volume of 26µl containing 1.44 X buffer G (Thermo Fisher Scientific, Inc., Waltham, MA, USA), 0.4 units of restriction enzyme and 8 µl of PCR product. Restriction digestion took place at 37 °C for 16 hours and digestion was halted by incubating at 65 °C for 20 minutes. Results were visualised in 2% agarose gel after 45 min electrophoresis at 140V in 0.5 X TAE (Tris-acetate-EDTA) buffer using peqGREEN staining at 1:25,000.

Data Analysis

Due to the skewed distribution of the catches, data were log-transformed prior to averaging. Means were detransformed (i.e. geometric means) for presentation (Figure 2.4). Data analysis was undertaken with the R statistical programming language (The R Foundation 2016). The significance of differences in catches was analysed using a general linear model with a negative binomial distribution. The MASS package in R (Ripley and others 2016) was used.

Results

2.4.1 Site recruitment

Sampling regions are shown in Figure 2.3. It was not possible to find drained farmland in the South West area sampled, so 2 more exposed hillside sites were chosen as a comparison (sites 18 and 19, at altitudes of 120m and 114m respectively: Table 2.2)

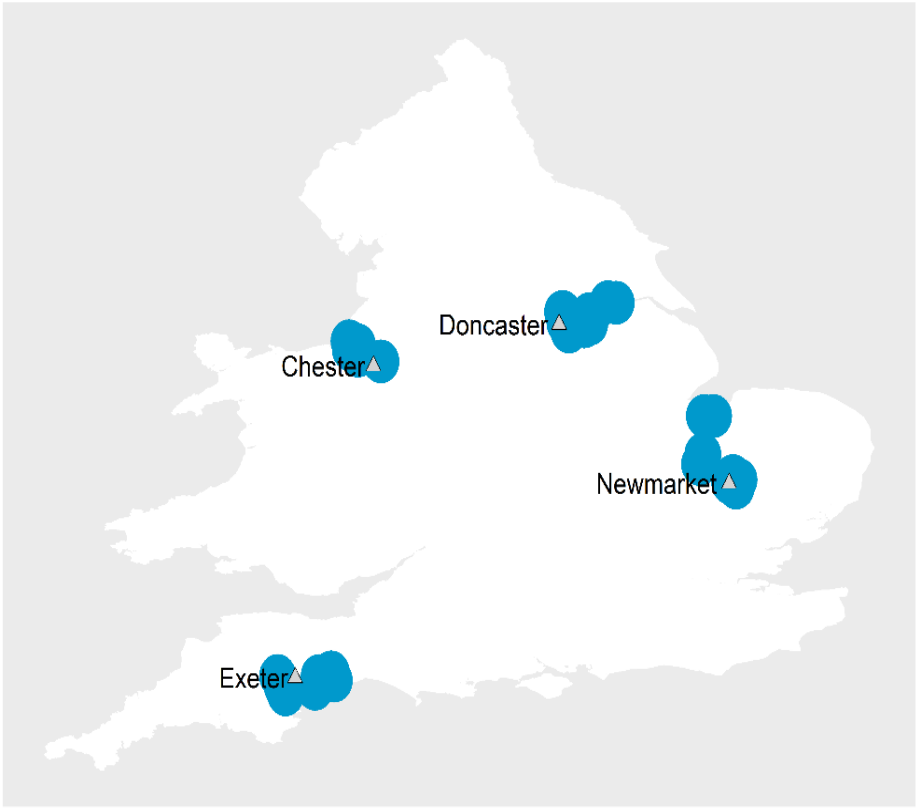

Figure 2.3 Map showing locations of sampling regions (Chapman et al., 2016)

		Most abundantly caught mosquito species							
Location Number and Region	**Habitats**	*Anopheles claviger*	*Anopheles plumbeus*	*Culiseta annulata*	*Ochlerotatus caspius*	*Ochlerotatus detritus*	*Ochlerotatus punctor*	**Other**	**Total**
NW 1	D	0	0	12	0	0	0		12
NW 2	U	6	0	7	24	1	0	*UA – 5*	43
NW 3	D	5	0	0	0	0	0		5
NW 4	U,S	0	1	12	3	53	0	*UA - 5*	74
NW 5	W	0	1	5	0	2	0	*OCA* - 3	11
NW 6	W	0	0	8	3	17	0		28
NW 7	S	0	0	14	1	176	0	*UA - 4*	195
NW 8	W,S	3	11	12	4	85	0	*UA - 4*	119
NE 9	U	0	0	1	0	0	0		1
NE 10	W,D	16	0	15	2	0	10	*OCA – 3, CR – 2, UA- 9 AV – 3,*	60
NE 11	W	0	0	6	0	0	0		6
NE 12	W,U	1	0	20	0	0	0	*CuS– 1, CR - 1*	23
NE 13	S	8	0	2	19	0	0		29
NE 14	S	5	0	0	3	1	0	*CR - 3*	12
NE 15	D	6	0	15	0	0	0	*AnM – 1, CR – 1UA- 1*	24
NE 16	U	3	0	2	0	0	0		5
SW 17	W	1	0	2	0	0	0	*AnM - 1*	4
SW 18	H	0	0	0	0	0	0		0
SW 19	H	0	0	0	0	0	0	*CxP – 1*	1
SW 20	W,S	3	0	2	0	4	0	*CxP – 1*	10
SW 21	S,U	0	1	0	8	0	0	*CxP – 1, UA- 8*	18
SW 22	W,U	0	0	0	0	1	2		3
SW 23	W	0	13	1	0	0	0		14
SW 24	W	0	1	1	0	0	0	*CxP – 1*	3
SE 25	U	0	0	0	0	0	0	*CxP – 1*	1
SE 26	W	0	0	1	0	0	0	*OD* - 1	2
SE 27	W,U	0	0	0	0	0	0	*CR - 1*	1
SE 28	W	0	0	6	0	0	0	*OR* - 3	9
SE 29	D	0	0	3	1	0	0	*OR* - 2	6
SE 30	S	0	0	4	33	155	0		192
SE 31	S	1	0	2	0	2	0		5
SE 32	D	0	0	1	0	0	0		1

Table 2.2 Adult mosquito species and number trapped in mosquito magnet
W – Woodland, D – Drained Farmland, S – Saltmarsh, U – Urban
UA – Unidentified Aedes spp., OCA – Oc. cantans, CuS – Cs. subochrea, CR – Cq. richiardii, AnM – An. maculipennis, CxP – Cx. pipiens, AV – Ae. vexans, OD – Oc. dorsalis, OR – Oc. rusticus

2.4.2 Host Seeking Adults

A total of 917 adult mosquitoes of 14 species were caught over a total of 285 trapping days over the 32 locations (Table 2.2). The geometric mean catch for each mosquito magnet

trapping period (approx. 72 hours) was 3.7 (SD 3.4), across all locations and seasons. Totals caught were 487, 217, 160 and 53 in the areas sampled in the NW, SE, NE and SW respectively.

For locations given one habitat classification, the geometric mean catch (9 days across 3 sampling periods) from a Mosquito Magnet was 6.9 (SD 5.90), 3.8 (2.5), 6.1 (3.3) and 36.5 (5.2) for premises associated with woodland, urban, drained farmland and saltmarsh habitats respectively (Figure 2.4).

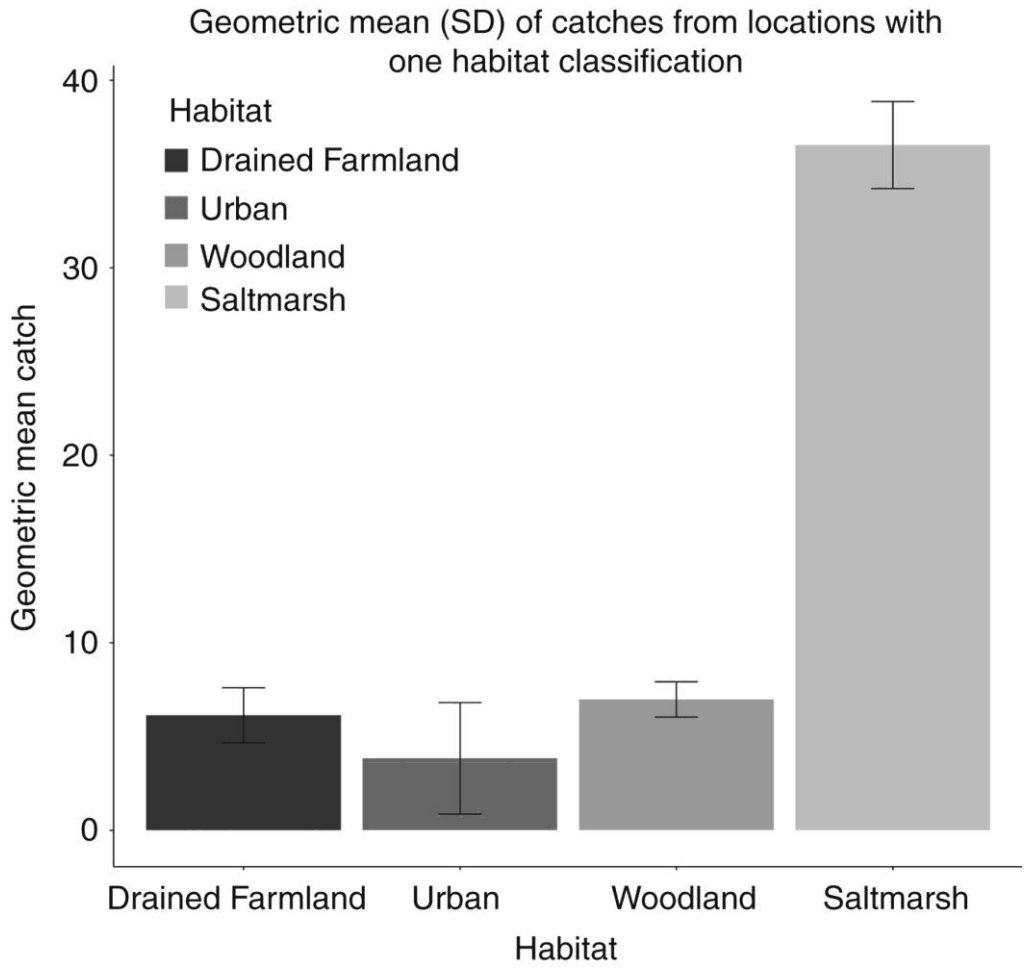

Figure 2.4 Geometric mean of total catch per location for each habitat type (locations only included if given 1 habitat classification) (Chapman et al., 2016).

The most trapped species was *Oc. detritus* with a total of 499 adults caught. All three sites with total catches > 100 were associated with the saltmarsh habitat of this species.

The second most trapped species was *Cs. annulata,* with 154 adults caught. *Cs. annulata* had the highest presence and was trapped on 75% (24/32) of sites.

Total catch was highest in September (Figure 2.5), and the difference in catch was significantly higher (P<0.005) than that in May and that in June/July. The total mosquito number from all locations was 679 with a geometric mean of 5.6 (SD 5.1) per location. One site was not sampled (location 19, Table 2.3) in September 2015 due to loss of the propane canister. A number of specimens could not be identified positively to species level due to trap damage, and are recorded as unidentified *Aedes spp*.

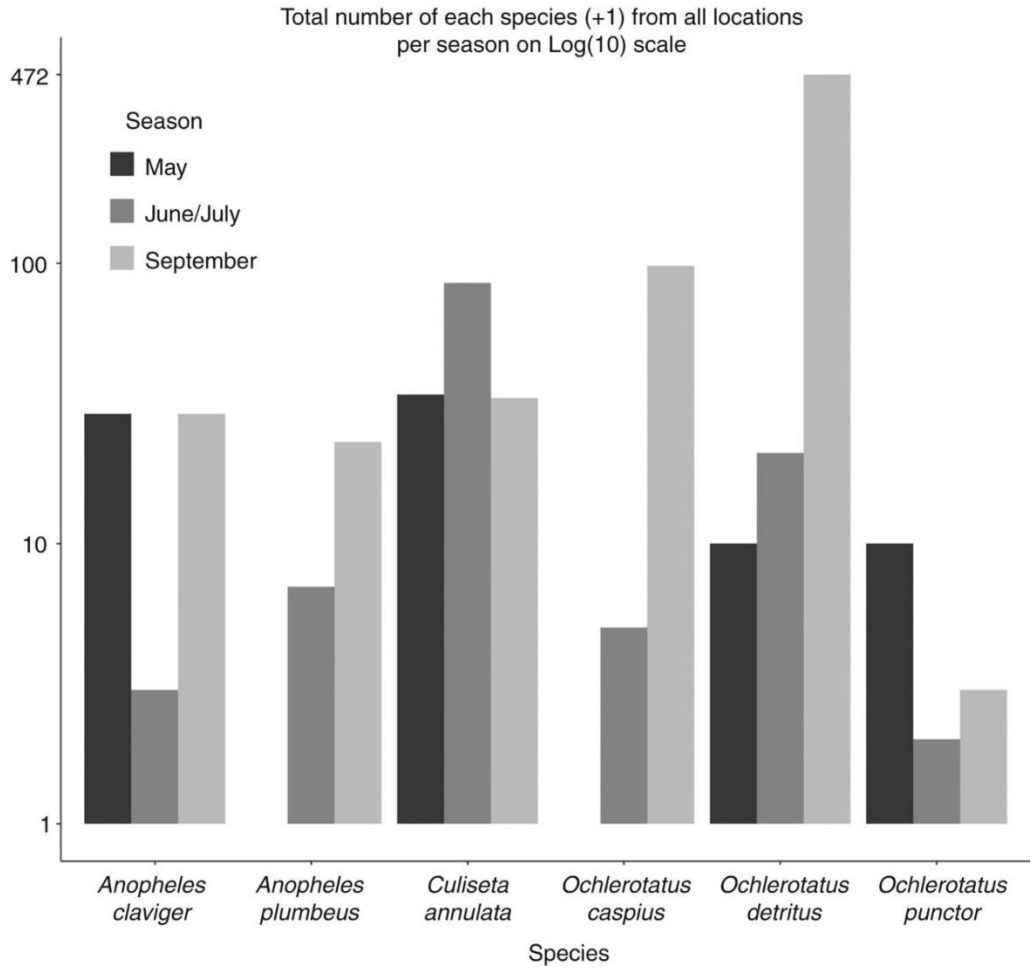

Figure 2.5 Total adult catches by season for each of 6 most abundant species (Chapman et al., 2016).

No mosquitoes were trapped whilst feeding on horses. Only 3 blood-fed mosquitoes were trapped, all were part-fed individuals caught in the Mosquito Magnet, of which 2 were *Oc. detritus* and one was *Cs. annulata.* One mosquito *(Oc. caspius)* was sampled landing on a human host.

2.4.3 Resting Adults

Sampling of resting mosquitoes was unsuccessful. No mosquitoes were found in the red-box traps.

2.4.4 Immature Mosquitoes

Immature mosquitoes were recovered by dipping of water sources on 23 of 32 premises (71.9%). A total of 61 samples containing mosquito larvae or pupae were collected from a variety of water sources including ditches, buckets and water butts, tyres, ruts, muck heaps, pools and ponds.

Cx pipiens s.l., Cx. torrentium, Cs. annulata/alaskaensis/subochrea, Cs. fumipennis, Cs. morsitans, Oc. caspius, An. claviger and *An. maculipennis s.l.* were captured using dipping techniques (Table 2.3).

Location	Cx. pipiens	Cx. torrentium	Cx. pipiens / torrentium[a]	Cs. annulata / subochrea / alaskaensis	Other species
NW2				✓	
NW3					*An. claviger*
NW4		✓			*Cs. morsitans*
NW7	✓		✓		*An. claviger*
NW8		✓		✓	
NE9	✓				
NE10	✓		✓	✓	*Oc. caspius*
NE12	✓	✓			
NE13	✓		✓	✓	
NE16	✓		✓	✓	
SW17	✓	✓	✓	✓	*Cs. fummipennis*
SW19		✓	✓	✓	
SW20	✓		✓		
SW21	✓	✓	✓		
SW22	✓	✓	✓	✓	
SW23	✓		✓		
SW24	✓	✓	✓		*An. claviger*
SE25	✓		✓		
SE27		✓	✓	✓	
SE28					*An. maculipennis s.l.*
SE30	✓	✓	✓		
SE31		✓	✓		
SE32	✓		✓		

Table 2.3 Larval species sampled on each location.

a - Not all *Cx. pipiens / torrentium* samples could be differentiated, due to financial constraints.

The majority of samples were from artificial containers with small amounts of water, such as tyres. Therefore, on most occasions, samples from each container were less than 500 ml, so it was not considered appropriate to state the numbers sampled, nor was it possible to

compare larval numbers across sites. Larval samples were used to identify the presence of a species rather than its relative abundance.

A selection (due to financial constraints) of larvae identified morphologically as *Cx. pipiens/torrentium* were further identified by molecular methods for each location. Of the 23 sites from which samples were obtained, *Cx. pipiens* larvae were identified from 15 (65.2%) of locations, *Cx. torrentium* from 11 (47.8%). Both species were found in 5 (21.7%) of these 23 locations. Both *Cx. pipiens* and *Cx. torrentium* larvae were obtained from at least 2 sites in all four regions.

Cs. annulata/alaskaensis/subochrea larvae cannot be differentiated morphologically and were obtained at 9 (28.1%) of the 32 sites. Due to the rarity of *Cs. alaskaensis* and the relative abundance of *Cs. annulata* it is likely that these are *Cs. annulata*. Considering both juveniles and adults, *Cs. annulata* were present at 27 (84.4%) of the 32 sites.

Discussion

This study described the first survey of mosquito species on multiple equine premises in the UK. This has demonstrated the presence of several mosquito species which are candidate vectors. Commonly found mosquito species on equine premises during this study included *Oc. detritus, Oc. caspius, Cs. annulata, Cx. pipiens s.l., Cx torrentium, An. claviger, An. plumbeus* and *Oc. punctor.* Although mosquito density could be considered low at most of the sites sampled, this can be partly explained by the fact that the spring of 2015 was relatively dry for all of the regions except the North West (Met Office, 2016). The species trapped in the current study are all considered mammalophilic or bite both birds and mammals, with the exception of *Cx. torrentium* which is strongly ornithophilic (bird-biting). Three European studies provide evidence that *Cx. pipiens s.l.* found in rural areas will bite mammals, including horses (Balenghien et al., 2008; Börstler et al., 2016; Schönenberger et al., 2016). Although not all of these studies differentiated *Cx. pipiens* form *pipiens* from *Cx. pipiens* form *molestus* the study of (Börstler et al., 2016) records a significant number of *Cx. pipiens* form *pipiens* with mammalian blood meals.

Eleven of the sixteen species found on equine premises during this study are laboratory competent vectors of or are implicated in, naturally occurring disease cycles for at least one arbovirus affecting horses (Table 2.1). Potential risk to the UK from these viruses is discussed in Chapter 1.

An important aspect of this study is that we trapped very few blood fed mosquitoes: just three in the Mosquito Magnet and none by other methods. This begs the question of whether the mosquitoes present at equine premises in the UK only rarely feed on equines, or whether they feed but were not caught. A number of factors suggest that the latter is the most likely explanation: (i) the Mosquito Magnet is designed to trap host-seeking rather than blood fed adults; (ii) many of the premises had other potential hosts present (humans, cattle, small mammals) indicating that the low number of trapped blood-fed mosquitoes cannot be attributed to the specific avoidance of equids; (iii) in pilot work in September 2014, mosquitoes *Cs. annulata, Oc. Caspius and Oc. detritus* were directly observed by the author feeding on horses and in addition in the field-repellency study (Chapter 5) *Oc. detritus* and *Cs. annulata* were shown to feed regularly on horses; and (iv) most of the species caught in this study have been reported, in other studies, to feed on horses and/or transmit arboviruses to horses.

A large sampling effort and high mosquito densities are required to maximise trapping of blood-fed mosquitoes. The number of sites included in this study dictated that sampling effort on each site was necessarily lower than that of other recent studies (Brugman, 2016). However, all the species sampled in this study, with the exception of *Cx. torrentium*, and *Cs. morsitans* have been shown to bite equines (Table 2.1), and four of the six most abundant species in adult catches have been shown to bite horses in the UK either in previous studies or in work conducted for this thesis. Further work would be required to investigate the feeding rate of UK populations of these mosquitoes on horses, and host bait catches (Schönenberger et al., 2016) would seem most likely to provide useful information.

The comparatively high numbers of *Oc. detritus* and *Oc. caspius* caught on some saltmarsh associated sites are consistent with previous studies and reports of significant nuisance biting (Clarkson and Setzkorn, 2011; Medlock et al., 2012a; Medlock and Vaux, 2013) and confirms that there is significant potential for host-vector interaction between these species and horses. These two species are competent vectors of WNV (Blagrove et al., 2016; Vermeil et al., 1960). Detailed, high-resolution information regarding horse and mosquito species distributions is lacking (Iacono et al., 2013). However, using previously published horse distribution data at postcode scale (Boden et al., 2012; Iacono et al., 2013) and saltmarsh distribution (Adnitt et al., 2007), in combination with mosquito species records, several coastal areas of England appear worthy of further investigation for host-vector interaction potential. These areas have high horse density, saltmarsh presence and records of *Oc. detritus* and *Oc. caspius*, (National Biodiversity Network, 2016a, 2016b; The Walter Reed Biosystematics Unit, 2014) and include the Severn Estuary, South Devon coast, the

South Coast of England from Swanage to Chichester and the Dee and Mersey estuaries. Two of these areas were sampled during this study: Wirral (Dee estuary) and the South Devon coast.

The finding that the WNV vector *Cx. pipiens* was common on equine premises with suitable water sources is expected, as this species has a widespread distribution in the UK (Medlock et al., 2005; Medlock and Vaux, 2011), but this study confirms that suitable container habitats are commonplace on equine premises. Horse owners should be encouraged to be aware of potential container habitats and avoid their presence, particularly if or when increased risk of transmission of equine or human arboviruses in the UK is predicted, for example WNV extension into Northern France, or incursion of new viruses into Europe. *Cx. torrentium* is a major enzootic (wildlife) vector of Sindbis virus in Scandinavia (Hesson et al., 2015) and may, therefore, be capable of a similar role in the transmission of other arboviruses. *Cx. pipiens* and *Cx. torrentium* were found on a number of occasions in all four regions, suggesting that *Cx. torrentium* may be more prevalent in the North of England than previously recognised (Medlock et al., 2005).

One of the most interesting results to emerge from the current study was the presence of *Cs. annulata* on the majority of sites (27/32). It was also the second must abundant species in Mosquito Magnet samples. Whilst *Cs. annulata* is known to have a widespread distribution in the UK (Medlock et al., 2005) this study provides evidence of the potential for host–vector interaction with UK equines. *Cs. annulata* has recently been demonstrated to be vector competent for WNV (M. Blagrove, unpublished) and as the species bites both birds and mammals including horses (Schönenberger et al., 2016), it, therefore, has potential to transmit arboviruses from avian reservoirs and hence serve as a 'bridge vector'. Combined with its ability to breed in a variety of water sources and presence on most sites sampled, this makes it an important species for further study.

Mosquito Magnets are a commonly used trap in Europe for surveillance. They catch almost all mammalophagic species of mosquito, catch more species than other systems and in greater numbers (Hutchinson et al., 2007). Red box traps were used in the current study to attempt to trap blood-fed mosquitoes, however, no mosquitoes were captured. Similar but larger red box traps have been successful in capturing *An. maculipennis s.l.*, *Culiseta annulata* and *Culex spp*. in England (Brugman, 2016). Knock-down catches were also unsuccessful. There were few buildings present on sampled sites considered suitable, due to ventilation gaps allowing mosquitos to escape. No mosquitoes were observed resting inside of buildings, despite inspection of at least 2 separate livestock buildings on each site visit (3-4 visits per location per season). Therefore, aspiration methods were not attempted. Based

on this work surveillance on equine premises in the UK should utilise Mosquito Magnets and larval sampling.

The number of sites sampled in this study was greater than previously published mosquito sampling studies in the UK and studies on European equestrian premises (Boukraa et al., 2016; Kampen et al., 2015). However, no comparison between seasons is possible as premises were sampled only in 2015.

In conclusion, the current study has demonstrated that horses in the UK are at risk of attack from a wide variety of mosquito species, several of which are known to be vectors of equine arboviruses in affected countries. It has also highlighted a number of mosquito species which should be investigated with regards to vector competence and effectiveness of protection measures for equines.

The results of this study suggest that mosquito species presence is determined mainly by local mosquito breeding habitat, rather than equine host availability or management factors. However, biting of horses may be affected by practices such as the use of repellents, rugs and masks, building design, and duration and timing of grazing, as well as the presence or absence of container habitats. In the event of increased risk of equine arbovirus transmission (cases in Northern France, new virus incursions in Europe or cases in the UK) education of horse owners in reducing transmission risk would be vital. A targeted information campaign using the veterinary profession and a variety of media would be necessary. Ideally, in this scenario pharmaceutical companies should also be encouraged to apply for UK licensing of vaccines against relevant arboviral diseases.

Appendix to Chapter 2

For each sampling location, the approximate area of the appropriate habitat was measured using QGIS™ and the UK Land Cover Map 2007 (Burns, 2014).

Habitat designation in this study	Land Cover Map Habitat Designations
Urban	Urban Suburban
Saltmarsh	Saltmarsh
Woodland	Broadleaved Woodland Coniferous Woodland
Drained Farmland	Fen, Marsh and Swamp Bog

Table 2.4 Land Cover Map 2007 habitats used to analyse land use around sampling locations.

Unfortunately, it was not possible to identify drained farmland and measure ditch length per km^2 using this method, and this was therefore not possible due to financial constraints.

For each location the area of each habitat was calculated using the Mosquito Magnet location as the centre of circles with radii: 500m, 1km, 2km, 5km, 8km. These distances were chosen due to the variation in flight distances for mosquito species. For example, woodland species such as *Ae. cinereus* may have flight distances of less than 500m, whereas others, such as the saltmarsh mosquito *Oc. detritus* may have flight distances of greater than 8km (Table 1.6). Habitat results were not further analysed due to the numbers of mosquitoes sampled.

		500m				1km				2km				5km				8km			
Location	Habitats	Woodland	Urban	Saltmarsh	Fen / Bog	Woodland	Urban	Saltmarsh	Fen / Bog	Woodland	Urban	Saltmarsh	Fen / Bog	Woodland	Urban	Saltmarsh	Fen / Bog	Woodland	Urban	Saltmarsh	Fen / Bog
NW 1	D	13645.21	21119.8	0	0	235447	381745	0	0	641300	4376769	0	0	3547447	34982449	0	0	7940272	80630038	5943538	0
NW 2	U	0	370357.6	0	0	66311	1789321	0	0	435825	5760358	0	0	3972465	28473981	19193	0	5477028	55804930	1860635	0
NW 3	D	0	34393.86	0	0	0	303950	0	0	66748	641706	0	0	758833	8952946	0	0	5092497	36619451	3587549	0
NW 4	U,S	0	438319.7	20154	0	163747	1282635	698653	0	527400	4388893	3492716	0	2615658	11088686	10331622	0	6777363	38405276	20040985	0
NW 5	W	40880.21	0	0	0	66311	12689	0	0	652245	585176	0	0	4131780	21140869	1156669	0	7359385	48716142	20522597	0
NW 6	W	55101.54	125721.2	0	0	114998	760473	0	0	250845	2953965	0	0	3275955	34982449	14877884	0	6936453	40703856	24703847	0
NW 7	S	10589.09	20006.16	0	0	86904	280138	254342	0	234369	2669638	3395863	0	1809155	11386282	14982869	0	7560876	35230062	24392684	0
NW 8	W,S	116944.8	6106.165	0	0	360159	122164	0	0	928748	1684447	776201	0	2388605	8413004	17026596	0	6641273	40689722	24082714	0
NE 9	U	1982.442	0	0	0	56544	20362	0	0	340031	54874	0	0	5139021	2042921	209040	0	11699463	14137206	468639	13833
NE 10	W,D	34819.84	14686.69	0	0	114892	13741	0	0	882659	280689	0	0	4312137	2085352	115294	3674720	11831558	7913949	0	9997428
NE 11	W	130210.7	22499.62	0	0	517206	22500	0	0	21	7	0	0	4138543	2371622	0	0	20372210	22814991	0	2013517
NE 12	W,U	270878.7	87481.21	0	0	793719	859750	0	18169	1779532	5685033	0	18169	6454312	27291322	467881	383069	15815532	48163589	248009	1157629
NE 13	S	25059.99	8008.225	0	0	44241	129373	0	0	12192726	965790	904409	0	1278081	1564120	3875727	1005924	4351941	6007624	4461892	1256039
NE 14	S	0	133077.1	0	0	18593	289603	12795	0	30370	344370	712784	0	547292	3162243	2152627	485082	6150294	9692215	3899012	721097
NE 15	D	6985.424	42774.84	0	0	113222	532026	0	0	313582	1230784	0	0	10085188	10274141	689580	689580	7746338	6525138	31040	14002695
NE 16	U	75624.68	7129.914	0	0	211516	143423	0	0	655262	1033759	0	15	2710753	4149742	6646	1428171	8690536	5810132	0	8357817
SW 17	W	117505.2	59888.21	0	0	505552	116313	0	0	1876163	932754	0	0	13424583	5324553	0	0	91500326	25939072	41437	0
SW 18	h	10231.4	33580.89	0	0	453609	87407	0	0	2356749	113577	0	0	20977866	273727	0	0	66719198	6363547	0	0
SW 19	h	5498.301	0	0	0	209508	87407	0	0	957887	273369	0	0	7828071	692863	0	0	36014194	4481178	0	0
SW 20	W,S	245732.9	79656.04	0	0	742061	68027	0	0	3214994	1120510	0	0	7694614	10928378	200003	0	12857015	16108481	358883	0
SW 21	S,U	0	520987.6	0	0	364	1950849	0	0	161617	5918389	5607	0	5521028	22431758	200739	0	56052023	57214321	864354	0
SW 22	W,U	62208.32	78903.06	0	0	438935	660920	0	0	1460557	1353603	0	0	5521028	22431758	0	0	17875911	8589638	0	0
SW 23	W	166728.1	28680.86	0	0	722217	38778	0	0	2634469	1120510	0	0	11327582	5342337	0	0	21607770	5328107	0	0
SW 24	W	310861.5	0	0	0	630804	40320	0	0	2068329	159410	0	0	11723358	4371062	0	0	23205618	7751395	11969	0
SE 25	U	23183.92	188584.1	0	0	77974	1370800	0	0	578557	4271247	0	0	4681779	7390095	0	0	12089021	12508684	0	0
SE 26	W	77059.89	10015.49	0	0	284404	29436	0	0	1192182	68426	0	0	5557250	3284616	0	0	12126426	16073622	0	0
SE 27	W,U	105352.5	11020	0	0	312712	95546	0	0	536325	369026	0	0	6453378	1524177	0	0	13707379	7779691	0	0
SE 28	W	137976.7	82093.74	0	0	371045	278606	0	0	906482	555485	0	0	6584853	2629361	0	0	15594904	10260274	0	0
SE 29	D	28593.51	0	0	530444	47765	17137	0	886240	134566	68240	0	1626579	420802	341308	0	4736799	1242302	3506554	0	6917034
SE 30	S	326720.8	9377.573	0	0	1278703	57519	0	0	4403149	105828	0	0	15445489	3204831	3837833	0	24099586	10423709	7754914	0
SE 31	S	130446.4	28550.94	0	0	845107	103288	0	0	2390470	731935	0	0	44868254	49818567	9287580	1646264	28293507	5113630	2437517	0
SE 32	D	0	62305.11	0	0	0	246481	0	0	64094	0	0	1501	619887	2688435	0	2266067	1247246	7033245	0	4317989

Table 2.5 Area (m^2) of each habitat within 500m, 1km, 2km, 5km and 8km of each Mosquito Magnet.

3 SURVEY OF UK HORSE OWNERS

Survey of UK horse owners' knowledge of equine arboviruses and disease vectors

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Abstract

Increased globalization and climate change have led to concern about the increasing risk of arthropod-borne virus (arbovirus) outbreaks globally. An outbreak of equine arboviral disease in northern Europe could impact significantly on equine welfare, and result in economic losses. Early identification of arboviral disease by horse owners may help limit disease spread. In order to determine what horse owners understand about arboviral diseases of horses and their vectors, we undertook an open, cross-sectional online survey of UK horse owners. The questionnaire was distributed using social media and a press release and was active between May and July 2016. There were 466 respondents, of whom 327 completed the survey in full. High proportions of respondents correctly identified photographic images of midges (71.2%) and mosquitoes (65.4%), yet few were aware that they transmit equine infectious diseases (31.4% and 35.9%, respectively). Of the total number of respondents, only 7.4% and 16.2% correctly named a disease transmitted by midges, and mosquitoes, respectively. Only 13.1% and 12.5% of participants identified specific clinical signs of African horse sickness (AHS) and West Nile virus (WNV), respectively. This study demonstrates that in the event of heightened disease risk educational campaigns directed towards horse owners need to be implemented, focussing on disease awareness, clinical signs and effective disease prevention strategies.

Introduction

Globalization and climate change have led to increasing concern over the risk of arthropod-borne virus (arbovirus) outbreaks in northern Europe (Durand et al., 2013; Medlock and Leach, 2015). There is evidence of increasing risk to equids in the UK and other areas of Europe that are currently free from equine arboviruses, and surveys of equine premises in the UK have demonstrated the presence of several species of mosquitoes that are known to be vectors of equine arboviruses (Chapman et al., 2016). Midges, which act as vectors for African horse sickness (AHS) are known to be widespread on UK equine premises (Robin et al., 2014).

Arboviral disease can appear and spread very rapidly, and new viruses may occur, as demonstrated by the Schmallenberg outbreak in ruminants that occurred in 2011 and spread across the UK (King et al., 2015). It has been stated that horse owner awareness of clinical signs would be key in limiting an outbreak of AHS in the UK (Sabirovic et al., 2008b). Early recognition of an equine arboviral disease and measures to limit its spread are essential in minimising rates of mortality and morbidity, and duration of a disease outbreak. For example, an outbreak of AHS that first started in central Spain in 1987 was initially not recognised, as local veterinary surgeons and horse owners were not aware of the key clinical signs of disease. The resultant disease outbreak spread over three countries and lasted over 4 years resulting in the death of approximately 1,400 equids in Spain alone (Rodriguez et al., 1992). An outbreak of AHS in Asia caused the deaths of over 300,000 horses from 1959-1961 (Mellor, 1993). It has been estimated that the cost to the UK government of an AHS outbreak could be £4-35 million, depending on the scale the outbreak (Gosling et al., 2012), and in the Netherlands, total costs have been estimated at 272-516 million Euros (Robin et al., 2016). In addition to AHS, horses in northern Europe are at potential risk from West Nile virus (Bessell et al., 2016) and possibly other mosquito-borne arboviruses, such as Eastern equine encephalitis virus (EEEV), Western equine encephalitis virus (WEEV), Venezuelan equine encephalitis virus (VEEV), and Japanese encephalitis virus (JEV) (Durand et al., 2013; Pages et al., 2009).

Assessment of horse owners' knowledge about clinical signs of arboviral diseases, how disease is spread and whether vaccines are available to control or limit disease spread are important factors to consider as owner compliance with preventive and control measures would be required in the event of a disease outbreak (Kung et al., 2013; Manyweathers et al., 2017; Schemann et al., 2012). This information could inform education strategies directed at horse owners about the risk of disease and how to recognise clinical signs in affected horses, and could assist early recognition of disease, particularly in situations of heightened disease risk.

The aim of this study was to investigate UK horse owners' knowledge about insects that bite horses, methods by which insects are prevented from biting horses and knowledge of insect-borne viral diseases that affect horses. A priori, we hypothesised that horse owners would have limited knowledge of insect-borne diseases of horses and would use a variety of bite-protection methods with spray-repellents being most popular.

Methods

3.3.1 Data collection

A cross-sectional survey (Appendix C) of UK horse owners/carers was conducted using an online questionnaire tool (Survey Monkey, Survey Monkey Inc. Palo Alto, California, USA). To be included in the study, participants had to be currently caring for one or more horses in the UK and be over 18 years in age. The questionnaire was available online May 13th - July 27th 2016. The survey was posted on the study website and was promoted via social media using Facebook and Twitter and in a university press release. The survey link was posted to general equine discussion forums and promoted through relevant organisations such as the British Horse Society and local groups of The Pony Club, through equine charities such as The Horse Trust and The Donkey Sanctuary and through social media, such as Facebook, Twitter and websites, as deemed appropriate by the administrators of these media. Veterinary practices also posted promotion of the survey. Further dissemination relied upon users sharing posts about the survey.

The survey focused on mosquito and midge-borne diseases of horses and covered the following themes: awareness and knowledge of arthropod borne diseases of horses worldwide; attitude to and knowledge of insects on the premises where their horse is kept; opinions about vaccination and bite protection methods, including use of insect repellents. The questionnaire was piloted with ten horse owners and amended based on their responses and comments before release.

3.3.2 Data analyses

A descriptive analysis of the responses to each question was performed. Respondents were not forced to respond to any single question so in calculating percentages the denominator used (unless states otherwise) was the number of participants who answered subsequent questions. Therefore, in the case of participants who left an answer blank but answered further questions it was inferred that the question had been left intentionally blank. For open-

ended questions inductive coding (application of categories produced by looking for patterns in the responses, rather than categories decided upon prior to release of the survey) was applied before analysis (Thomas, 2006). Coding was undertaken solely by the first author. Comparisons between two sample proportions were tested using a 2 - sample z – test (Sergeant, 2016).

ETHICS

Ethical approval was granted by the University of Liverpool Veterinary Ethics Committee (VREC398).

Results

In total 466 surveys were completed and 70.2% (327) had been completed in full (defined as at least one question completed on all pages of the survey, and completion of all questions which could not be answered 'I don't know'). All responses were analysed.

ABILITY TO IDENTIFY INSECTS, AND INSECT NUISANCE

A total of 365 respondents (98.6%, n=370) stated they were aware of biting insects on the premises where their horse was kept (Table 3.1) including five respondents who additionally named arthropods that are not biting flies, such as spiders and hornets. In the free text answers, six respondents named ticks. Overall, 95.1% (352/370) of respondents named at least one biting fly (Mosquito, Midge, Stable fly, Horse fly, Gnat). The majority of respondents (331/465, 71.2%) were able to correctly identify a photographic image as a midge, and only 11 (2.4%) reported not having seen this insect before. Over half of respondents (284/434, 65.4%) were able to correctly identify a photographic image as a mosquito and only 14 (3.2%) reported not having seen one before. Of those respondents who answered 'yes' to whether they were aware of mosquitoes on their yard, 79.5% (105/132) were able to correctly identify mosquitoes, compared to 57.9% (135/233) of respondents who were not aware of mosquitoes on their yard ($P < 0.001$). A stable fly was correctly identified by 56.9% (235/413) of respondents and 49.4% respondents (195/395) correctly identified a horse-fly.

Insect	Respondents reporting they are aware of this insect on their yard (n = 367)	Number of these respondents correctly identifying insect
Mosquito	132 (36.0%)	105 (79.5%) 114* (86.4 %)
Biting Midge	322 (87.7%)	239 (74.2 %)
Stable fly	132 (36.0%)	100 (75.8 %)
Horse fly	294 (80.1%)	155 (52.7 %)

Table 3.1 Responses to awareness of biting insects present on the participant's yard, and the proportion correctly identifying images of these insects.

For each image options given were Mosquito, Biting Midge, Stable Fly, Horse Fly, Gnat, I have never seen this before, and I do not know. *including those using the term 'gnat' to describe a mosquito in the image.

Overall, 27.0% (99/367) respondents stated they were unaware of mosquitoes and 6.8% (25/367) of midges, on their yard. Figure 3.1 shows the distribution of participants who were aware of different biting flies on their horse's yard and provided postcode data. Participants were asked if they felt the insects caused a problem, not if they specifically caused a problem to the horse. Of 132 respondents who said they were aware of mosquitoes on their yard, 15 (11.4%) stated they did not cause a problem, 70 (53.0%) stated they caused a minor problem and 47 (35.6%) considered that they caused a moderate or major problem. Of respondents who were aware of midges on their yard, only 2.5% of participants (8/322) stated they caused no problem, whilst 65.5% of (211/322) participants felt they caused a moderate or major problem.

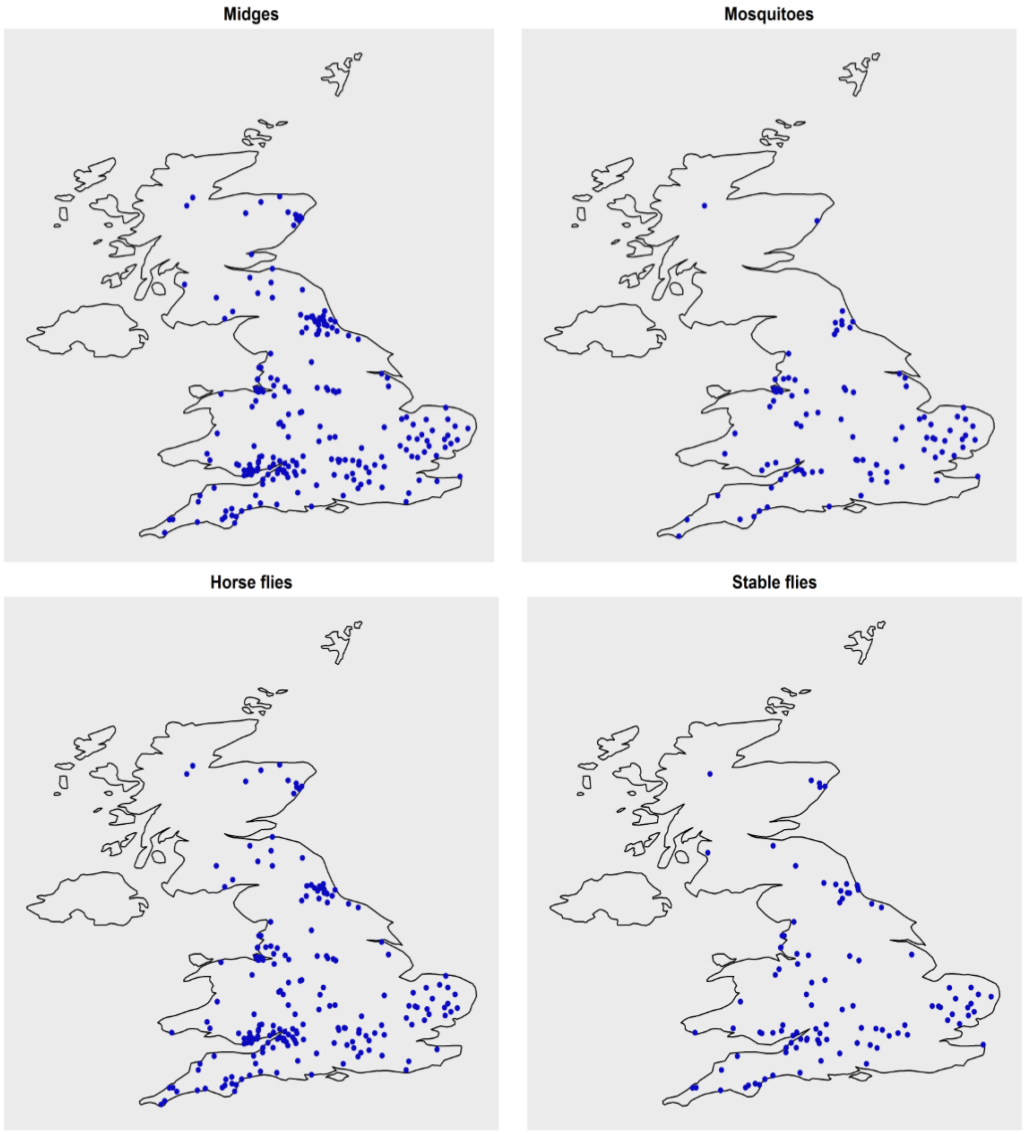

Figure 3.1 Distribution of yards on which participants reported that they were aware of each type of fly, (and provided geographical information).

KNOWLEDGE ABOUT INSECTS AND EQUINE DISEASE

Of 331 respondents who correctly identified midges, 31.4 % (104/331) stated that this type of insect could transmit infectious diseases to horses. Of respondents who correctly identified mosquitoes, 40.5% (115/284) believed they only caused allergic disease and 35.9% (102/284) believed that they transmitted infectious diseases in horses worldwide. However, only 7.4% (n=27) of all respondents (366) were able to correctly name a midge borne disease and 16.2% (59/365) a mosquito borne disease of horses. When asked to state any infectious disease transmitted by midges, to horses worldwide, only 27 of 366 respondents (7.4%) named African Horse Sickness. Other responses to this question were

sweet itch (6.6%; n=24) bluetongue (n=3), malaria (n=1), sarcoids (n=5) and WNV (n=2). When asked to state a mosquito-borne disease of horses, 59 out of 365 respondents (16.2%) correctly identified at least one disease. Most of the correct answers stated WNV only and a small number of respondents (2.7%, 10/365) cited other encephalitides including 'equine encephalitis' (n=5), Eastern equine encephalitis (EEE) (n=4) and Western equine encephalitis (n=4). None of the respondents mentioned Venezuelan equine encephalitis (VEE), Murray valley encephalitis (MVE), Ross River fever, or other mosquito-borne viruses. AHS was believed to be transmitted by mosquitoes by 9.3% (34/365) of respondents and 9.9% (36/365) cited that mosquito-borne diseases of other species affected horses. These were predominantly human diseases including malaria, Zika, Q-fever, and dengue. 'Malaria' was cited as a disease of horses transmitted by mosquitoes by 7.9% (29/365) of participants. However it should be noted that equine piroplasmosis, a tick-borne disease, is also known as equine malaria (Wise et al., 2014). Significantly more respondents were aware of AHS (72.5%, 261/360) compared with WNV (60.5%, 219/362) ($P = 0.002$). Figure 3.2 shows that the geographical locations of those who were aware of WNV and AHS were not distributed in one particular region. When asked specifically if West Nile Virus (WNV) can affect horses 83.1% (182/219) of those who responded to this question stated 'yes'.

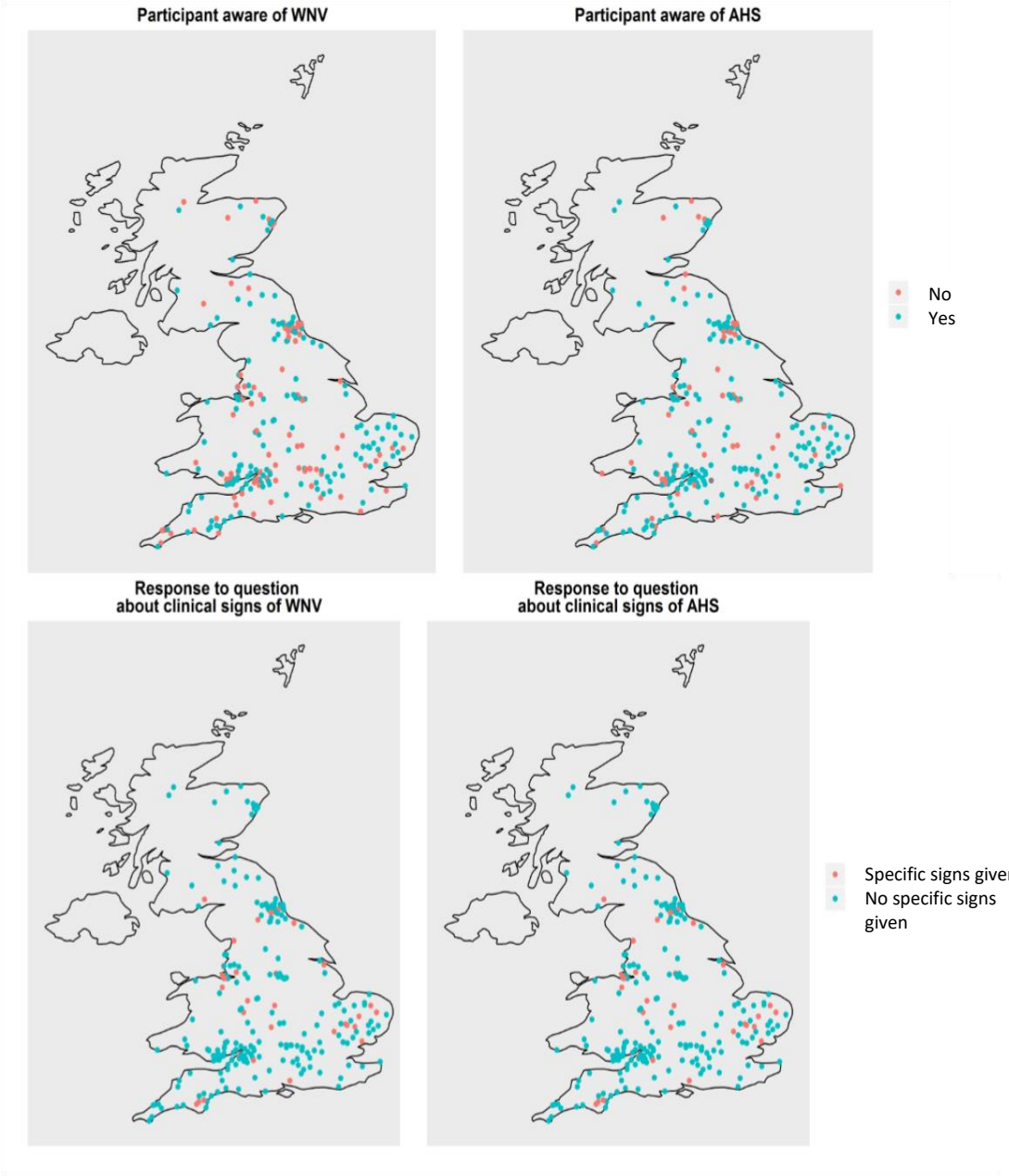

Figure 3.2 Approximate locations and responses of participants who answered questions about their knowledge of WNV and AHS (and provided geographical information).

CONSEQUENCES OF AN EQUINE ARBOVIRUS OUTBREAK AND CLINICAL SIGNS OF DISEASE

A summary of responses to statements about the possible consequences of an outbreak of either AHS or WNV in the UK, is provided in Table 3.2. When asked to list clinical signs of WNV only 21.1% (n=69) of all participants responded, despite 327 respondents submitting this page of the survey. Of those who responded, 59% (n=41) listed correct clinical signs such as neurological abnormalities, and 17.3% (n=12) stated non-specific signs, which were coded as pyrexia, depression and inappetence, and two respondents mentioned only ‘flu-like

symptoms'. Of all survey participants completing this page, the proportion who stated neurological signs in relation to WNV was 12.5% (41/327).

When asked to list the clinical signs of AHS, only 22.9% (n=75) of survey participants responded, out of 327 submitting this page of the survey. Of these, three responded that they were not aware of the clinical signs and 43 (13.1% of 327) provided at least one correct and specific clinical sign of AHS, such as foaming at the nostrils, respiratory compromise, or facial swelling. Again, the geographical distribution of those demonstrating knowledge of clinical signs for either disease was unremarkable (Figure 3.2). Only ten respondents (3.1%) stated death or collapse, despite the high mortality caused by this disease.

	WNV			AHS		
Statement	**True (n)**	**False (n)**	**I don't know (n)**	**True (n)**	**False (n)**	**I don't know (n)**
Disease could spread rapidly throughout UK (Respondents: WNV 326; AHS 326)	48.1% (157)	4.9% (16)	46.9 (153)	56.1% (183)	4.6% (15)	39.3% (128)
Many horses could become ill (327;326)	55.4% (181)	4.3% (14)	40.4% (132)	67.8% (221)	1.8% (6)	30.4% (99)
Horses could die from the disease (328;328)	52.7% (173)	2.1% (7)	45.1% (148)	66.5% (218)	0% (0)	33.5% (110)
Lots of horses * (more than 1,000) could die from the disease (327;324)	30.9% (101)	7.3% (24)	61.8% (202)	42.9% (139)	3.1% (10)	54.0% (175)
The government would ban movement of horses in affected areas (327;329)	36.7% (120)	6.7% (22)	56.6% (185)	47.7% (157)	7.6% (25)	44.7% (147)
A vaccination campaign would be necessary to prevent further spread (327;329)	30.3% (99)	5.8% (19)	63.9% (209)	39.8% (131)	7.3% (24)	52.9% (174)
Vaccination could be done immediately to protect horses (325;328)	17.2 (56)	11.4% (37)	71.4% (232)	15.9% (52)	16.2% (53)	68.0% (223)

Table 3.2 Summary of participant responses to statements regarding the consequences of an outbreak of AHS or WNV in the UK.

*participants were provided with the information that there are approximately 900,000 horses in the UK.

VACCINATION

Participants were asked if their horses were currently vaccinated against influenza, tetanus or other diseases. Overall, 97.9% (320/327) responded their horses were vaccinated for tetanus and 88.1% (288/327) for influenza. Only 16 stated their horse was vaccinated against other diseases, including equine herpes virus and grass sickness. One respondent stated their horse was vaccinated against WNV. In the event of a disease outbreak of WNV, most respondents to this question answered that they would have their horse(s) vaccinated against it (80.1%, 262/327), 57 respondents (17.4%) were unsure and eight respondents (2.4%) would not vaccinate. Participants were asked to write brief comments about factors that would make

them less likely to choose vaccination for WNV. Themes included concerns about side-effects and efficacy (51.4%; 76/148 respondents), lack of risk of disease (27.7%; 41/148), and cost (14.9%; 22/148). A few respondents (9.5%; 14/148) stated that there was nothing that would prevent them vaccinating their horse(s) in this situation. Of the five respondents who said they would not vaccinate their horses, four were concerned about side effects. Of the 43 respondents who were unsure about vaccinating and gave reasons, 56% (n=24) cited potential lack of risk as a barrier, while 41.9% (n=18) cited the balance between efficacy and side effects. Respondents who stated that they vaccinate their horse(s) against influenza were more likely to have them vaccinated against WNV, compared with those who do not vaccinate against influenza ($P < 0.001$). Due to the lack of a commercially available vaccine suitable for use in the UK, we did not ask about vaccination against AHS in the current study.

USE OF BITE PROTECTION METHODS

Participants were asked if they used repellents, fly masks or rugs at pasture, in the stable or when ridden (Figure 3.3). The majority of respondents (90.2%; 284/315) stated that they used repellents, 69.5% (219/315) used fly rugs and 71.7% (226/315) used fly masks or fringes. In addition, 10.5% (33/315) of respondents used stable barriers such as fly mesh to prevent insects entering and 35.2% (111/315) used wash-in insecticides on their horse, such as ‘Deosect’ (Cypermethrin 0.1% w/v).

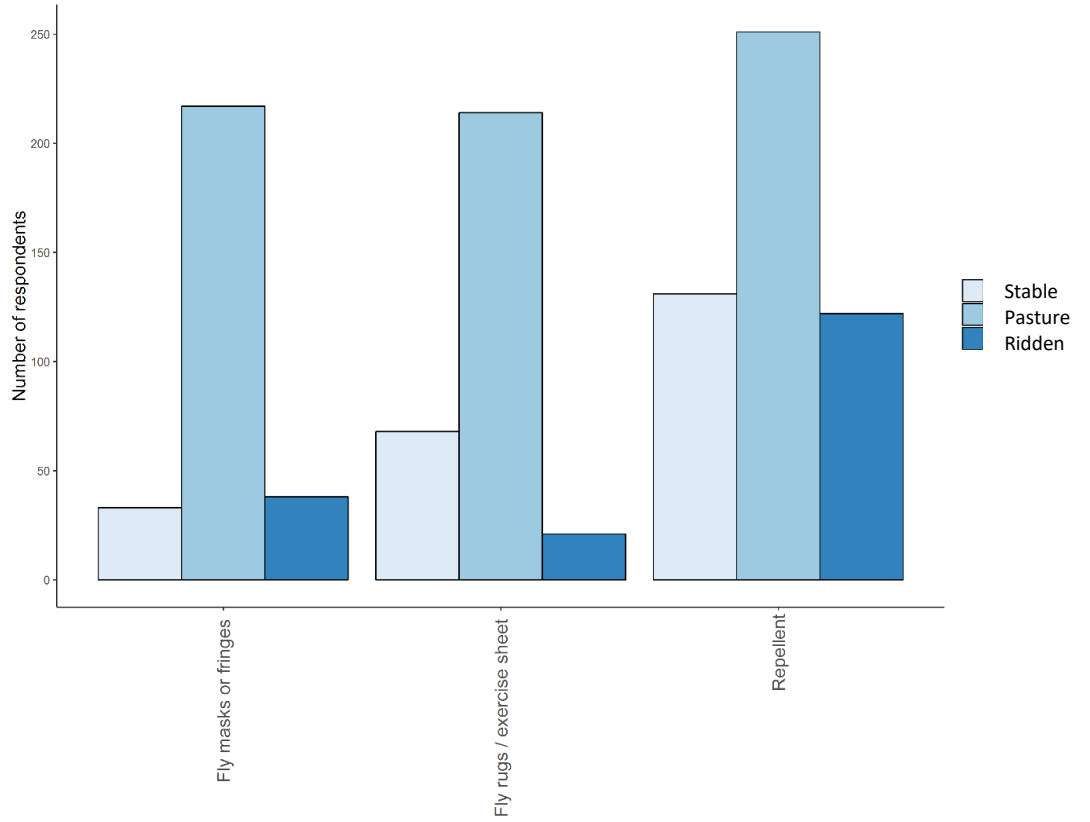

Figure 3.3 Number of respondents using insect bite protection methods, in the stable, at pasture, and when ridden.

Participants were asked to state which insect repellents they used on their horse(s). They were also asked about alternative bite protection methods in a subsequent question in which some respondents cited products which are believed to have a repellent effect. Respondents' answers were cross-checked between the two questions to prevent double counting. There were 252 respondents in total and N,N-Diethyl-meta-toluamide (DEET) and citronella were the most popular insect repellents used (Table 3.3). Other methods respondents used to try to reduce insect bites included feeding garlic (n=15), turmeric (n=1), or yeast (n=1), turning horses out at specific times of the day (n=2), use of insecticide or repellent impregnated tags (n=5), fly traps in stables and fields (n=4), permethrin impregnated rugs (n=1), and fans in stables (n=1). When asked an open question about comparative effectiveness of bite-protection methods against all of the biting flies listed, 15 of 46 (32.6%) respondents stated specifically that nothing seemed to work against horse flies.

SOURCES OF INFORMATION

When participants were asked where they would seek information about insect control, 65.1% (203/312) stated that they would seek advice from their veterinary surgeon and 55.8% (174/312) stated they would use internet sources. Other common answers included tack shop staff (28.2%; 88/312) and other people keeping horses at the same premises (29.5%;

92/312). When asked if a disease outbreak might motivate them to seek information from a different source, 295 participants responded and 63.0% (186/295) stated 'yes' to this question. A total of 85.3% (266/304) said that they would seek information about insect control from a veterinary surgeon in the event of an outbreak of disease in the UK. Of the respondents stating they would change or add to sources of information, 17.2% (32/186) expected to be able to obtain information from governmental or industry sources. A small number of respondents (n=6) mentioned they expected the government to issue specific guidelines on insect control in the event of an outbreak.

Discussion

This survey provides important evidence that UK horse owners currently have poor awareness of equine arboviral diseases, including clinical signs of disease, consequences, and controls which might be imposed in the event of a disease outbreak. Given the increasing risk of an equine arboviral disease occurring in northern Europe and potentially the UK, it is important that the veterinary profession has a good understanding of horse owners' level of knowledge about clinical signs of disease, ways in which a specific equine arboviral disease is spread and controlled. The profession should be able to provide correct, current, evidence-based information to horse owners in the event of a disease outbreak of equine arboviral disease.

Culicoides biting midges can induce insect-bite hypersensitivity ("sweet-itch") in horses (Wilson et al., 2001), a common disease in the UK (McCaig, 1973). Therefore, horse owners might be expected to have knowledge about how to identify and control midges. Most study respondents were able to correctly identify different flies that bite horses, including midges and mosquitoes, but few horse owners (around a third) were aware that midges and mosquitoes could transmit diseases to horses.

Horse owners' knowledge regarding equine disease transmitted by mosquitoes and midges was poor: many respondents were aware of AHS, WNV, WEE or EEE or 'equine encephalitis' but none mentioned Venezuelan equine encephalitis, Murray valley encephalitis, Ross River fever, or other less well-known arboviruses. Many respondents were also unaware of likely consequences of an outbreak of WNV or AHS. For control of AHS in accordance with the Disease Control Strategy of Great Britain, vaccination during an outbreak and banning horse movements are preferable to culling (except for clinically affected animals). Only 42.9% of respondents answered 'True' for the statement that 'lots of

horses could die from AHS' and just under 70% believed that 'many horses could become ill' (Table 3.2). Just under half of respondents were aware that movement bans may be implemented during an AHS outbreak. These results indicate a lack of awareness of the potentially devastating consequences of an AHS outbreak, to both equine welfare and the equine industry as a whole. Over a third of respondents in this study believed that the government would ban horse movements in the event of an outbreak of WNV, and just over a third believed that a vaccination campaign would help prevent spread. Neither of these measures are appropriate for control of WNV since the horse is a dead-end host and subclinical infections are common (although vaccination protects the individual horse). The majority of remaining respondents answered, 'I don't know' (Table 3.2).

There are inherent difficulties in using survey questions of this type i.e. using true or false responses for statements in this manner: responses may be biased towards agreement with the statements provided. It would have been preferable in this situation to use an open question; however, the decision was made to use true or false statements to act as a memory aid to participants, in case of partially recalled information. Particularly for these questions we assumed poor knowledge might be a problem, potentially leading to drop-out of participants, or free text answers which could not be coded. By biasing this question towards producing apparently greater knowledge, we can be more confident that lack of knowledge is unlikely to be overestimated: i.e. even lower levels of knowledge are likely in the general population than reported. Ideally, further investigation of such complex questions would benefit from qualitative research methodology based on interviews.

A report by DEFRA on the risk factors and potential likelihood of risk of introduction of AHS into the country states that "Awareness and familiarity of owners, keepers of horses and veterinarians with AHS clinical signs would facilitate early detection as a key limiting factor to potential wider dissemination of the disease in the UK" (Sabirovic et al., 2008b). Whilst the majority of participants responded that AHS could cause death, only a small proportion gave specific clinical signs for AHS or for WNV. This lack of specific knowledge is not surprising given that these diseases have never occurred in the UK but illustrates the need for dissemination of information to horse owners in the event of heightened disease risk. Online information on AHS is provided by The British Horse Society (BHS, 2014), the UK government (DEFRA, 2014) and by individual veterinary practices, but based on the lack of knowledge in the horse owning community demonstrated by this study, investigation into horse owner engagement with these sources is warranted.

Surprisingly, a higher proportion of UK respondents (80.1%) than in Kentucky, USA (66%) said they would vaccinate against WNV even though the disease is endemic in Kentucky

(Dalton, 2006), and only around 40% of UK leisure horses are vaccinated against influenza, which is present in the UK (Merial, 2016). However, respondents in the present study may have been biased towards horse owners with an interest in equine diseases and may, therefore, be more likely to vaccinate. The proportions of horse owners that stated they had their horses currently vaccinated, is comparable with another UK horse owner survey in which 78.8% and 87.6% of respondents reported that their horses were vaccinated against influenza and tetanus, respectively (Hotchkiss et al., 2007). The main barriers to vaccination against WNV were potential side effects of a vaccine or the balance between side effects and efficacy. This would be important information to convey to veterinary surgeons, to promote discussion with horse owners, and provide reassurance in the event of heightened disease risk to the UK equine population.

Perceived efficacy of fly repellents by horse owners is likely to centre on general reduction in fly nuisance around horses. Therefore, biting flies such as midges and horse flies probably have more impact on repellent choice, than mosquitoes in the UK. Most study respondents stated that they currently seek information on bite-protection from their veterinary surgeon. Therefore, it is important that veterinary surgeons have evidence-based information in order to provide correct advice, particularly in a situation of heightened disease risk (Middleton et al., 2014; Taylor et al., 2016). Of the active ingredients used by respondents in this study, icaridin and DEET have both been shown to have some repellent effect for horses against mosquitoes (Boehringer Ingelheim, 2010; Palmer, 1969). Evidence of repellent effect of products in this study are summarised in Table 3.3. However it should be noted that many studies show variable durations of effective protection against biting insects, few repellents have been tested in any livestock species and efficacy in human studies may not translate to protection for horses (Carpenter et al., 2008). Due to the small size of *Culicoides* midges, direct testing of repellency for horses is challenging (Page, 2016).

Ingredient / Repellent type	Total Responses (% of participant responses[c]) (n=252)	Evidence of repellency against mosquitoes	Evidence of repellency against *Culicoides*
N,N-Diethyl-meta-toluamide (DEET) (including Power Phaser)	89 (35.3%)	Yes [1,2]	Yes [3,4]
Citronella	48 (19.0%)	Variable[2]	No[5]
Power Phaser (DEET and IR3535)	36 (14.3%)	Yes[2]	Yes[4]
NAF product (active ingredient not identified)	25 (9.9%)	-	-
Home made	25 (9.9%)	-	-
Citridiol (also known as oil of lemon eucalyptus, citriodora, PMD)	24 (9.5%)	Yes[2,6]	Yes[7]
Neem oil	19 (7.5%)	Yes[6]	Yes[8]
Avon skin so soft (citronellol)	19 (7.5%)	No	No
Icaridin	8 (3.2%)	Yes[a 2,9]	Yes[10]
Tri-tec (cypermethrin and pyrethrins)[b]	3 (1.2%)	-	-
Coopers Fly Repellent (permethrin and citronellol)[b]	5 (2.0%)	-	-
Unintelligible	25 (9.9%)	-	-

Table 3.3 Insect repellents used on horses as reported by study respondents, and evidence for repellent efficacy in studies upon humans.

a -Studies on both horses and humans. b - Usefulness of topical insecticides is unclear because of their inability to prevent blood-feeding, although treatment of infected horses may subsequently kill vectors that have blood-fed (Papadopoulos et al., 2010). c – participants who did not respond to this question, or did not state that they used repellents were not included

1. Palmer (1969)
2. (Lupi et al., 2013)
3. Braverman and Chizov-Ginzburg (1998)
4. (González et al., 2014)
5. Page (2016)
6. (Maia and Moore, 2011)
7. (Trigg and others, 1996)
8. (Blackwell et al., 2004)
9. Boehringer Ingelheim (2010)
10. (Carpenter et al., 2005)

There were some limitations to the study that are inherent in open, internet-based surveys. It was not possible to obtain a randomised representative sample of horse owners and therefore it was not possible to estimate a response rate. The inference that any questions left blank (unit non-response) were left intentionally blank may lead to bias, however this was considered a trade-off as non-response due to lack of knowledge is common (Okafor, 2010). It was considered that requiring responses in a survey investigating limited knowledge may lead to unacceptable loss of participants through forcing them to choose 'I don't know', an answer they may not find comfortable. Sampling bias is also likely due to the requirement for internet access, and self-selection of horse owners with an interest in the topic of the questionnaire. Respondents may be biased towards the more media literate and, therefore, possibly, younger horse owner. Respondents were not asked for demographic information such as their age or sex, however, a previous online survey of horse owners in Great Britain (Boden et al., 2013) reported that 95.2% of respondents were female and 51.6% were under 45 years old. The British Equestrian Trade Association's National Equine Survey 2015 (British Equine Trade Association, 2015) reported that females represented 74% of the riding population. It may have been useful to enquire what other countries the participant had resided in: those having lived in South Africa could be expected to have greater

knowledge of African horse sickness, for example. The number of responses to the study is likely to have precluded meaningful analysis of this type of information. In our study respondents were not instructed to limit responses to one per household, so clustering of knowledge, or lack thereof was possible. However, the majority of respondents supplied partial postcodes. Clustering within postcode districts was not apparent and where only town was supplied the maximum people giving the same town was two. This occurred only in four locations.

To our knowledge this the first survey of UK horse owners that has been conducted to determine awareness of insects that transmit equine arboviruses, clinical signs of disease and disease control methods. Whilst the majority of respondents were able to identify insect vectors such as mosquitoes and midges, most were unaware of diseases that they may transmit. In addition, most study respondents were unable to provide specific clinical signs of WNV and AHS, and few stated that these diseases may cause death of affected horses. A variety of methods were reported to be used to repel insects from biting horses, but in many cases, there is no published evidence of efficacy. The veterinary profession was stated as the key source of information about insect control and would be vital in the event of a disease outbreak in disseminating evidence about clinical signs of disease, methods of insect control and vaccination. Based on this study, should an equine arbovirus disease outbreak occur in northern Europe, it would be important for the UK veterinary profession to be able to quickly implement a horse owner education campaign and for veterinary surgeons to be able to provide accurate information about clinical signs of disease and methods of disease prevention and control.

4 VECTOR COMPETENCE OF PALEARCTIC MOSQUITOES FOR EQUINE ARBOVIRUSES

Abstract

There has been no evidence of transmission of mosquito-borne arboviruses of equine or human health concern, to equine or human hosts, to date in the UK. However, in recent years there have been a number of outbreaks of viral diseases spread by other vectors in northern Europe, and of mosquito-borne viral diseases in southern Europe. These events, in conjunction with increasing rates of globalisation and climate change, have led to concern over the future risk of mosquito-borne viral disease outbreaks in northern Europe and have highlighted the importance of being prepared for potential disease outbreaks. The aim of this study was to test the competence of wild-caught British mosquitoes for arboviruses that are pathogenic to both humans and equines: *Ochlerotatus detritus* for Venezuelan equine encephalitis virus (VEEV) and Ross River virus (RRV), *Culiseta annulata* for VEEV and Japanese encephalitis virus (JEV), and *Culex pipiens* and *Ochlerotatus punctor* for JEV. Colony mosquitoes were challenged with these viruses for comparison: *Aedes albopictus* with JEV, RRV and VEEV, *Aedes aegypti* with JEV and VEEV and *Cx. quinquefasciatus* with JEV. Laboratory competence was demonstrated at varying efficiencies for all the virus-vector pairs involving British mosquitoes, although both *Oc. detritus* and *Cs. annulata* were shown to be inefficient vectors of VEEV. *Oc. detritus* was an efficient vector of RRV. *Cs. annulata*, *Oc. punctor* and *Cx. pipiens* demonstrated efficient transmission of JEV. Strikingly, *Cx. pipiens* was shown to be an efficient laboratory vector of JEV at 18 °C. *Aedes albopictus* [MAL] demonstrated efficient transmission of RRV and transmission of JEV. *Aedes aegypti* [RECIFE] was shown to transmit JEV. A small proportion of *Cx. quinquefasciatus* [MUHEZA] were shown to transmit JEV. The evidence that *Cx. pipiens* is an efficient vector at relatively low temperatures may necessitate re-assessment of the risk of virus emergence for JEV in temperate regions where it is abundant.

Introduction

Globalisation and climate change are expected to change the level of risk for emergence of disease in different regions of the world. In the last 50 years, the geographical range of a number of arboviral diseases has increased, including Zika, dengue, chikungunya and West Nile. Whilst the emergence in Europe of dengue and chikungunya has been associated with *Aedes aegypti* and the invasive mosquito *Aedes albopictus* (Papa, 2017), West Nile virus is mainly spread by *Culex* mosquitoes and the expansion in its range has demonstrated vector competence of previously naïve mosquito species or populations (Sardelis et al., 2001, 2002, Turell et al., 2001, 2005). Other emerging diseases that affect equines include Peruvian horse sickness virus (Attoui et al., 2009) and Bunyamwera virus (Tauro et al., 2015, 2016). Both are mosquito-borne viruses that have emerged as fatal equine diseases, in Peru and Argentina respectively, within the last 20 years.

There has been much discussion of the risk of equine arbovirus introduction to Europe in the last 10 years (de Vos et al., 2012, 2017; Durand et al., 2013; Faverjon, 2015). There has also been significant interest in predicting the risk of autochthonous transmission of emerging viruses of humans, and of future distributions of major human pathogens (Ho et al., 2017; Perkins et al., 2015) and invasive or widely distributed vectors, for example, *Ae. albopictus*, *Ae. aegypti* and *Oc. japonicus japonicus* (Caminade et al., 2012; Irwin et al., 1991; Melaun et al., 2015). Modelling has also been used to estimate the risk of outbreaks of livestock disease such as bluetongue in Europe (Guis et al., 2012), and on seasonal population dynamics of UK populations of mosquitoes for the purposes of disease risk estimation (Ewing et al., 2016). The mosquito-borne diseases of horses generally have complex transmission cycles involving wildlife hosts and 'bridge vectors' which bite more than one type of host and cause infection in clinically affected hosts such as horses. Therefore, these viruses have a broad host and vector range. To estimate the risk of autochthonous transmission (post-introduction) of these viruses in unaffected countries it is necessary to consider potential native vectors. Therefore, to produce useful outcomes from modelling it is imperative that potential vectors which are already present and potentially abundant in the region of interest are investigated for vector competence.

Japanese encephalitis virus has a broad geographical distribution and although it has been generally associated with pig and rice production in proximity, outbreaks in Oceania demonstrate that this is not an absolute requirement, at least for non-endemic transmission. JEV has several secondary vectors (Impoinvil et al., 2013) as well as the main vector *Cx. tritaeniorhynchus*, and has been identified in numerous species of wild-caught mosquitoes including *Cx. pipiens* (Kim et al., 2015; Su et al., 2014).

Venezuelan equine encephalitis virus has a highly complex transmission cycle involving regular mutation of the virus, facilitating transmission to horses through broadening of the vector and host ranges. Epidemic strains of VEEV produce high viraemic titres in horses which are the main amplification host. In some circumstances, it is believed that humans may produce levels of viraemia capable of infecting mosquito vectors (Morrison et al., 2008). Therefore, epidemic VEEV strains could reach Europe through movement of people or pets as well as improper control of horse export during an outbreak in the Americas. Endemic VEEV could be imported through exotic pet transport (Durand et al., 2013; Pages et al., 2009), although there is likely to be a low risk of onward transmission from each import in this case, without the epidemic mutation.

In Australia, Ross River virus is maintained in a transmission cycle between mosquito vectors and marsupial hosts. However, a large outbreak occurred in the South Pacific in 1979-1980 (Rosen et al., 1981), providing evidence that regions without native marsupial hosts may be at risk, and that humans may be capable of maintaining transmission. Recently there has been a suspicion that there is low-level endemic transmission on Pacific islands without marsupials and with a limited number of potential vertebrate host species. This raises the possibility that the potential for RRV to spread globally may be much greater than previously thought (Lau et al., 2017).

In Chapter 2, equine premises were sampled for potential mosquito vectors, and species of mosquito with the possibility of significant contact with the equine host in the UK were described. These species were considered priorities for vector competence testing in the context of risk to the UK.

In this study laboratory vector competence of British populations of Palearctic mosquito species for three equine arboviruses: JEV, VEEV and RRV, was assessed. None of the field-caught mosquito-vector combinations tested here have been tested previously except for JEV and *Cx. pipiens*, which was examined at a significantly lower temperature than previously (de Wispelaere et al., 2017). All the virus-vector pairs tested in the present study are also significant to human health: the viruses cause morbidity in affected regions, and the vectors tested have broad host ranges. Therefore, the information presented here is of importance to both public and veterinary health risk assessment.

Methods

5.3.1 Mosquitoes

Experiments were conducted on adult mosquitoes that were collected as larvae or pupae from marshland, woodland ponds, or container habitats on the Wirral Peninsula, North West

England. Immature mosquitoes were reared in ambient conditions in water collected from their larval habitat, supplemented with tap water as necessary. Where supplementary food was required Brewer's Yeast was provided. Adults were allowed to emerge and mate in 30 × 30 × 30 cm BugDorms (BugDorm, Taichung, Taiwan). Adults were kept in ambient conditions until exposure and were offered 10% sucrose solution on cotton wool.

Colony mosquitoes were used for comparison and were: *Ae. albopictus [MAL] (obtained from ARCTEC at the London School of Hygiene and Tropical Medicine), Ae. aegypti [RECIFE] and Cx. quinquefasciatus [MUHEZA] (from the Liverpool School of Tropical Medicine)*. These mosquitoes were reared in an insectary at 27 °C with a 12:12 light: dark photoperiod and 70 % relative humidity (RH). *Cx. quinquefasciatus were reared and provided by Henrietta Carrington Yates, Ae. Aegypti were reared by Jonathan Thornton, and stock Ae. albopictus were maintained by Amalia Anthousi at the Liverpool School of Tropical Medicine.*

5.3.2 Viruses

Viruses used were the JEV strain CNS138-11 (Solomon et al., 2003), *cultured and titre assayed by the Brain Infections Group, University of Liverpool in Vero* cells; RRV (catalogue number 0005281v) and VEEV P676 (catalogue number 0605153v) *cultured and titre assayed by Public Health England, Porton Down, Surrey*, in Vero cells. Final virus titre in blood was $1x10^6$ pfu/ml for JEV, 5.6×10^6 $TCID_{50}$/ml for RRV, and 9.5×10^6 pfu/ml for VEEV. Titres were limited by the stock concentration provided by the respective institutions (measured by those institutions, using plaque assay (JEV, VEEV) or endpoint dilution assay $TCID_{50}$ (RRV)).

5.3.3 Infection and Transmission

At 10 - 21 days post emergence, female mosquitoes were transferred into 1-litre polypropylene Dispo-safe containers (The Microbiological Supply Company, Luton, UK), with a fine mesh covering and were starved of sugar for 24 hours. In a containment level 3 facility, in a glovebox (custom model – Coy Laboratory Products, Grass Lake, Mississippi, USA) they were then allowed to feed for up to 3 hours, in low light conditions, on heparinised human blood (NHS transfusion service, Speke) or for colony mosquitoes, defibrinated horse blood (TCS Biosciences, Buckingham, UK) containing the virus. A Hemotek membrane feeding apparatus (Discovery Workshops, Lancashire, UK) heated to 39 ˚C was used with the membrane provided by the manufacturer. Immediately before use this was placed inside the researcher's sock for 15-20 minutes, to impart human odour, and encourage feeding. After the blood meal was removed, unfed mosquitoes were removed

from the cage using a mechanical pooter (Watkins & Doncaster, Leominster,UK) (to avoid exposing fed mosquitoes to triethylamine, which can cause loss of the blood meal), killed with Virkon spray, and discarded. Blood-fed females were incubated at 18 ˚C, 21 ˚C, or 24 ˚C for wild caught mosquitoes and 27 ˚C or 28 ˚C for colony mosquitoes. Mosquitoes were maintained at this temperature for 7-35 days and were provided with cotton pads soaked with 10% sucrose which were replaced twice per week. On the day of testing, mosquitoes were immobilised with triethylamine (FlyNap, Carolina Biological Supply Company, Burlington, North Carolina, USA), and their saliva was extracted by inserting each mosquito's proboscis into a capillary tube containing mineral oil for 30 minutes. Each mosquito and its expectorate were placed in a separate 1.5ml microcentrifuge tube containing 200µl TRIzol reagent (Thermo Fisher Scientific), kept at room temperature for 2 hours to inactivate virus and then stored at -20 ˚C.

5.3.4 Measuring Infection and Transmission

The field-caught mosquito species used in this study are temperate species and have not been tested previously, for vector competence for the viruses described, (with the exception of *Cx. pipiens* and JEV). The aim was to provide evidence of transmission, or lack thereof, (rather than detailed extrinsic incubation period data). Therefore, temperatures chosen were relatively low to reflect UK temperatures, and time points were chosen to give a broad range. Plaque assay, or other direct quantification of virus using cell culture for Hazard Group 3 Pathogens was not available, and therefore semi-quantitative RT-qPCR was used to estimate viral RNA quantities in mosquito saliva and carcasses. Viral RNA in samples may have a greater copy number than viable virions, however the effect is likely to be minimal for saliva samples and low virion numbers are required in mosquito saliva to cause infection in susceptible hosts.

RNA EXTRACTION

RNA was extracted using TRIzol reagent: samples were homogenised in 200 µl TRIzol using a P1000 pipette tip. They were then incubated at room temperature for 5 minutes. 40 µl chloroform was added and the samples were shaken for 15 seconds. Samples were centrifuged at 16400 rcf at 4 °C for 15 minutes. The aqueous upper layer was removed and was transferred to a new 1.5 ml Eppendorf tube, and 100 µl isopropanol was added. The tube was inverted several times to mix the contents and was then incubated at -20 °C for >10 minutes (or overnight). The sample was subsequently centrifuged at 16400 rcf at 4 °C for 10 minutes. The isopropanol was removed with a P200 pipette pressed against the bottom of the tube. Next, 1 ml of 75% ethanol was added and the sample was vortexed to suspend the

pellet before centrifuging at 16400 rcf at 4 °C for 5 minutes. The ethanol was removed and another 1 ml of ethanol was added. After vortexing, the sample was again centrifuged at 16400 rcf at 4 °C for 5 minutes. Again, the ethanol was then removed. The sample was then centrifuged briefly and the remaining ethanol was removed using a P20. After air drying the RNA pellets for 5-10 minutes RNase-free water was added (20 µl for mosquito body samples and 10 µl for saliva samples). RNA was dissolved by pipetting each sample up and down several times. Samples were stored at -20 °C for up to 14 days before cDNA generation.

cDNA GENERATION

cDNA was generated using Superscript™ Vilo™ (Thermo Fisher Scientific). Each 20 µl reaction consisted of 4 µl Superscript™ Vilo™ MasterMix, 6 µl RNase-free water, and 10 µl of sample. PCR plates were incubated at 25 °C for 10 minutes, then 42 °C for 90 minutes and the reaction was terminated at 85 °C for 5 minutes. cDNA was stored at -20 °C.

Virus	Sense Primer	Probe	Antisense Primer	Reference
VEEV	5'TCCATGCTAATGC YAGAGCGTTTTCGC A3'	5'Fam-TGATCGARACGGAGGTR GAMCCATCC-Tamra3'	5'TGGCGCACTTCC AATGTCHAGGAT3'	(Vina-Rodriguez et al., 2016)
RRV	5'TTGCCGGTGGGTA GAGAGAA3'	5'Fam-ACCACACTTTGGCGTAG AGC-Tamra3'	5'TCTGGCGGTGTA TGCATGTC3'	This study[a]
JEV	5' ATCTGGTGYGGYAG TCTCA3'	5'Fam-CGGAACGCGAWCCAGG GCAA-Tamra3'	5'CGCGTAGATGTT CTCA GCCC3'	(Lindahl, 2014; Pyke et al., 2004)

Table 4.1 Primer and probe sets for the TaqMan assays

a – Designed using Primer-BLAST (Ye et al., 2012)

TAQMAN QRT-PCR

TaqMan (Thermo Fisher Scientific) quantitate reverse transcription polymerase chain reaction (qRT-PCR) was used to detect the presence of viral RNA in the samples. Primer and probe sets are shown in Table 4.1.

TaqMan qPCR assays were performed in a reaction volume of 20 µl. The reaction contained 1 x TaqMan Gene Expression Master Mix (with ROX passive reference), TaqMan probe (500 nM for VEEV and RRV assays; 150 nM for JEV assay), primers (1 µM for VEEV and RRV assays; 400 nM for JEV assay) and 2 µl of cDNA or control substance.

Thermocycler conditions for VEEV and RRV assays were: 1 cycle of 95 °C for 10 minutes, then 45 cycles of 95 °C for 15 seconds, 55 °C for 30 seconds and 60 °C for 30 seconds. For the JEV assay these cycles were: 1 cycle of 95 °C for 10 minutes, then 45 cycles of 95 °C for

15 seconds, and 60 °C for 1 minute. Amplification and detection were performed using an Agilent Mx3005P qPCR System (Agilent Technologies, Santa Clara, California).

ANALYSIS

For each cDNA generation, a no-template control (nuclease-free water), and a positive control (viral RNA) were assayed. For each TaqMan assay, a positive control (cDNA generated from neat virus RNA) and negative controls (nuclease-free water, and cDNA generated from a mosquito infected with JEV for VEEV and RRV assays or infected with VEEV for JEV assays) were included.

For each virus, a standard curve for the PCR was generated using 3 replicates of 10-fold serial dilutions with a dynamic range of 7 logs using the stock virus (Table 4.2). JEV stock had been assayed at $1x10^9$ pfu/ml, VEEV at $9.5\ x10^9$ pfu/ml and the undiluted stock was used for JEV and VEEV. RRV stock had been assayed at $5.6\ x\ 10^9$ $TCID_{50}$/ml. An aliquot diluted to $2.2\ x\ 10^9$ of RRV was used. CT values were plotted against corresponding virus titre in Microsoft Excel to produce an equation and correlation coefficient (R^2) for the best fit line using Least Squares estimation (see Appendix). The efficiency of the TaqMan assay was calculated using the formula:

$$Efficiency\ (E_{AMP}) = -1 + 10^{(-\frac{1}{slope})}$$

Virus	Efficiency	Standard Curve Equation	R^2	Range of CT values produced in standard curve	Amplification factor
JEV	103.19%	y = -3.2477x + 43.108	0.9972	19.88-39.35	2.03
RRV	95.04%	y = -3.4467x + 44.284	0.9949	17.77-37.35	1.95
VEEV	91.66%	y = -3.5393x + 45.531	0.9978	17.80-38.61	1.92

Table 4.2 Parameters of the TaqMan assays.

The copy number of viral RNA in the stock virus was not known and therefore viral copy number cannot be estimated from CT value. Samples were considered positive for viral RNA if the CT value obtained from the sample was ≤40.

To aid the interpretation of CT values on plots, an 'estimated relative RNA quantity' is represented for each viral RNA, on a scale showing orders of magnitude – relative to a sample producing a CT value of 40 (see Appendix). It is important to note that the method used here is semi-quantitative and the scales presented on plots correspond to transformed CT values and not to absolute quantification of virus or RNA quantity (see Appendix for detailed explanation).

In this study, for percentage infection and transmission the denominator was the total number of mosquitoes successfully feeding on infected blood and surviving until the point of sampling. Infected mosquitoes were those in which the carcase tested positive for viral RNA. Mosquitoes for which expectorate tested positive for viral RNA, were considered able to transmit the virus. The transmission rate was the proportion of infected mosquitoes able to transmit the virus.

All statistical analysis was performed using the statistical programming language R (R Core Team, 2017). The difference in two proportions was analysed using Fisher's (fisher.test), the Shapiro-Wilks test was used to test whether data were normally distributed (shapiro.test), the Kruskal-Wallis rank sum test (kruskal.test) was used to test for significant differences in CT values between groups, and pairwise Mann Whitney-U tests (wilcox.test) with a Holm correction (Holm, 1979) was used to test for significant differences between each pair of groups.

Results

A total of 943 mosquitoes survived their respective incubation periods and were sampled. It was not considered appropriate to report mortality due to accidental mosquito death

(drowning caused by overzealous application of sucrose solution). A summary of sampled mosquitoes is presented in Table 4.3.

Species	JEV	RRV	VEEV
Oc. detritus	-	232	300
Oc. punctor	8	-	-
Cs. annulata	154	-	67
Cx. pipiens	18	-	-
Ae. albopictus	32	43	40
Ae. aegypti	16	-	3
Cx. quinquefasciatus	30	-	-

Table 4.3 Summary of mosquitoes exposed to virus and surviving until sampling.

5.4.1 Japanese Encephalitis virus

VECTOR COMPETENCE OF *CS. ANNULATA* FOR JEV

Cs. annulata collection from artificial containers at Ness Gardens produced usable numbers of adult females from late June to early September 2016. *Cs. annulata* was evaluated after challenge by ingestion with JEV and incubation at 21 ℃ and 24 °C at 3 time points (14 days, 21 days and 28 days). Infection data are summarised in Table 4.4.

Temperature (°C)	Timepoint (days)	Number of mosquitoes in sample (n)	Number infected	Number transmitting	% infected	% transmission	Transmission rate (%)
21	14	30	13	9	43.3	30.0	69.2
	21	35	20	15	57.1	20.0	75.0
	28	30	3	1	10.0	3.3	33.3
24	14	30	6	0	20.0	0.0	0.0
	21	24	4	0	16.7	0.0	0.0
	28	5	0	0	0.0	0.0	0.0

Table 4.4 Summary of infection and transmission rates of JEV in *Cs. annulata*.

% transmission – proportion of mosquitoes tested with JEV in saliva. Transmission rate – proportion of susceptible mosquitoes transmitting JEV

Estimated relative RNA quantities for these samples are presented in Figure 4.1.

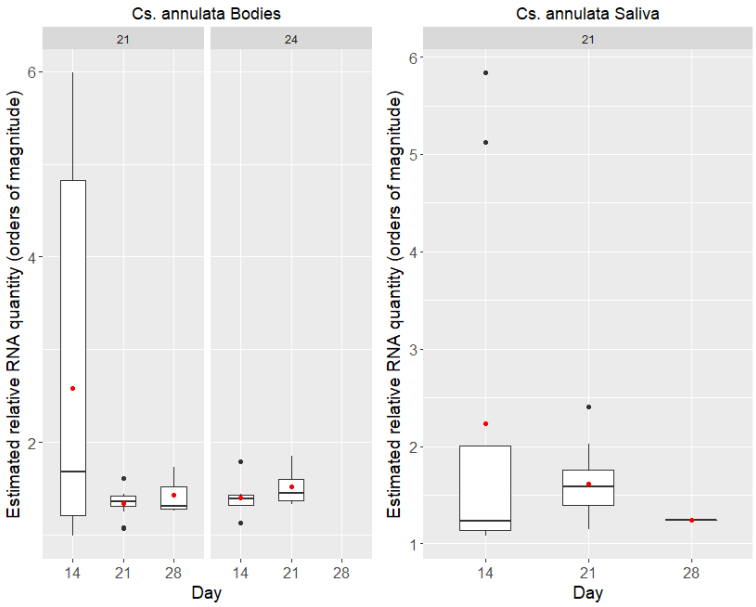

Figure 4.1 Range of estimated relative quantities of JEV RNA in samples from *Cs. annulata* bodies and saliva.

Box and Whisker plots – boxes indicate 2nd and 3rd quartiles, vertical lines upper and lower quartiles, and horizontal lines the median. Black points indicate outliers. Red points indicate mean values.

The trend in percentage infection and transmission is a reduction over time and reduced infection and transmission at 24 °C compared to 21 °C (Figure 4.2).

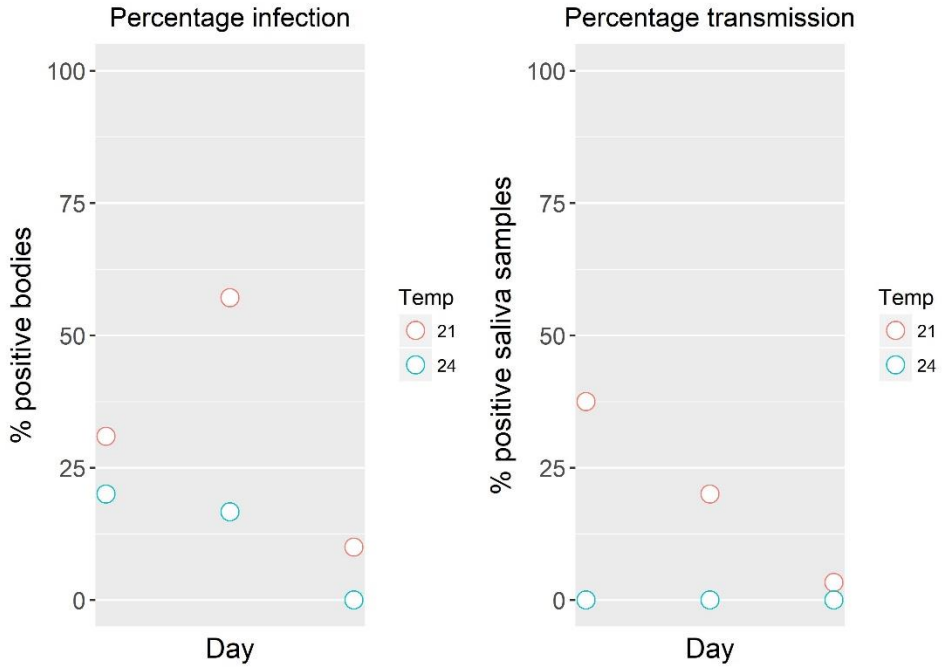

Figure 4.2 Proportions of *Cs. annulata* infected and capable of transmission.

VECTOR COMPETENCE OF *CX. PIPIENS* FOR JEV

A small number of *Cx. pipiens s.s.* collected on behalf of *and identified to species level* (Hesson et al., 2010) *by, Jenny Hesson, University of Liverpool*, became available for infection. These were tested at one temperature (18 °C) and one time point (21 days).

All 18 mosquitoes became infected, and 13 (72.2%) of these had viral RNA in saliva. Median CT values produced from mosquito bodies and saliva were 33.85 and 36.78 respectively (Figure 4.3).

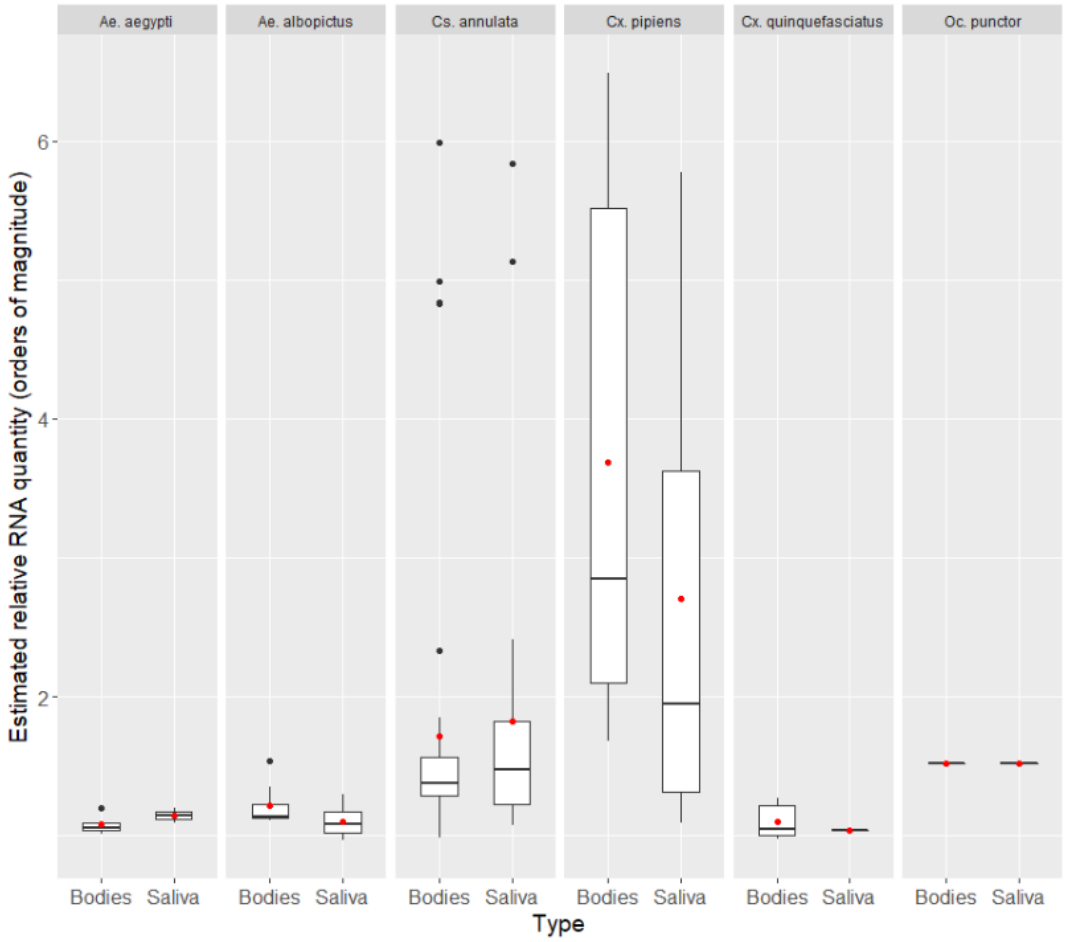

Figure 4.3 Range of estimated relative RNA quantities produced from all species (including all samples).

Box and Whisker plots – boxes indicate 2nd and 3rd quartiles, vertical lines upper and lower quartiles, and horizontal lines the median. Black points indicate outliers. Red points indicate mean values.

VECTOR COMPETENCE OF *OC. PUNCTOR* FOR JEV

Oc. punctor adult survival in captivity was poor. Of approximately 300 larvae, only 86 adult females were available at 14 days for infection. Of 35 blood-fed mosquitoes, 8 survived a 14 day incubation period. Significant mortality was apparently associated with handling. Therefore, only one temperature (21 °C) and one time point (14 days) were tested.

75% (6 of 8) samples demonstrated infection and 4 of these had viral RNA in saliva, giving a total transmission percentage of 50%, and 66.67% transmission from infected mosquitoes. Median CT values produced from mosquito bodies and saliva were 34.82 and 37.84 respectively (Figure 4.3).

VECTOR COMPETENCE OF COLONY MOSQUITOES FOR JEV

Ae. albopictus, Ae. aegypti and *Cx. quinquefasciatus* were challenged with JEV and incubated at 28 °C. *Ae. albopictus* and *Ae. aegypti* were sampled after 14 days and Cx. *quinquefasciatus* after 21 days. Infection data are summarised in Table 4.5.

Species	Timepoint (days)	Number of mosquitoes in sample (n)	Number infected	Number transmitting	% infected	% transmission	Transmission rate (%)
Ae. albopictus	14	32	13	8	40.6	25.0	61.5
Ae. aegypti	14	16	4	2	25.0	12.5	50.0
Cx. quinquefasciatus	21	30	6	1	20.0	3.3	16.7

Table 4.5 Summary of infection and transmission rates of JEV in colony mosquitoes.

% transmission – proportion of mosquitoes tested with JEV in saliva. Transmission rate – proportion of susceptible mosquitoes transmitting JEV.

Cx. pipiens produced higher estimated relative RNA quantities (lower CT values) in bodies and saliva than any of the other species, CT values were not normally distributed (Shapiro-Wilks test) and variances were significantly different between groups. A Kruskal-Wallis rank sum test indicated significant differences in CT values (including all time points) between species for bodies (χ^2 = 52.813, df = 5, P-value <0.001), and for saliva (χ^2 = 19.04, df = 5, P-value $\leq$ 0.01). Pairwise tests indicated that *Cx. pipiens* had significantly higher carcase estimated relative RNA quantities than all the other species. Due to the small sample sizes, the only significant differences in saliva samples were that both *Cx. pipiens* and *Cs. annulata* had significantly higher estimated relative RNA quantities than *Ae. albopictus* (P-value $\leq$ 0.05).

Pairwise Fisher's Exact test was used to compare proportions of infection and transmission between species (at their maximum, i.e. 14 days at 21 °C for *Cs. annulata*). Proportion infected for *Cx. pipiens* was significantly higher than for *Ae. albopictus* (P-value $\leq$ 0.05, *Ae. aegypti* (P-value $\leq$ 0.01), and *Cx. quinquefasciatus* (P-value <0.001). Proportion transmitting virus for *Cx. pipiens* was significantly higher than for any other species (P-value <0.001), except for *Oc. punctor*.

5.4.2 Ross River Virus

VECTOR COMPETENCE OF *OC. DETRITUS* FOR RRV

Oc. detritus were maintained at 21°C and 24 °C for 7, 14, 21, 28 or 35 days after feeding on a blood meal containing RRV. Infection data of *Oc. detritus* are summarised in (Table 4.6). Percentage transmission was highest after 7 days with an incubation temperature of 24 °C.

Temperature (°C)	Timepoint (days)	Number of mosquitoes in sample (n)	Number infected	Number transmitting	% infected	% transmission	Transmission rate (%)
21	7	33	23	9	69.7	27.3	39.1
	14	30	25	11	83.3	36.7	44.0
	21	30	2	0	6.7	0.0	0
	28	20	1	0	5.0	0.0	0
	35	10	0	0	0.0	0.0	-
24	7	22	16	11	72.7	50.0	68.8
	14	30	4	0	13.3	0.0	0.0
	21	30	0	0	0.0	0.0	-
	28	12	0	0	0.0	0.0	-
	35	15	0	0	0.0	0.0	-

Table 4.6 Summary of infection and transmission rates of RRV in *Oc. detritus*.

% transmission – proportion of mosquitoes tested with RRV in saliva. Transmission rate – proportion of susceptible mosquitoes transmitting RRV.

At both temperatures, by 21 days, both the percentage of mosquitoes able to transmit RRV and the proportion with detectable RNA in their bodies had dropped significantly compared to those at 7 days (P-value <0.05) (Figure 4.4).

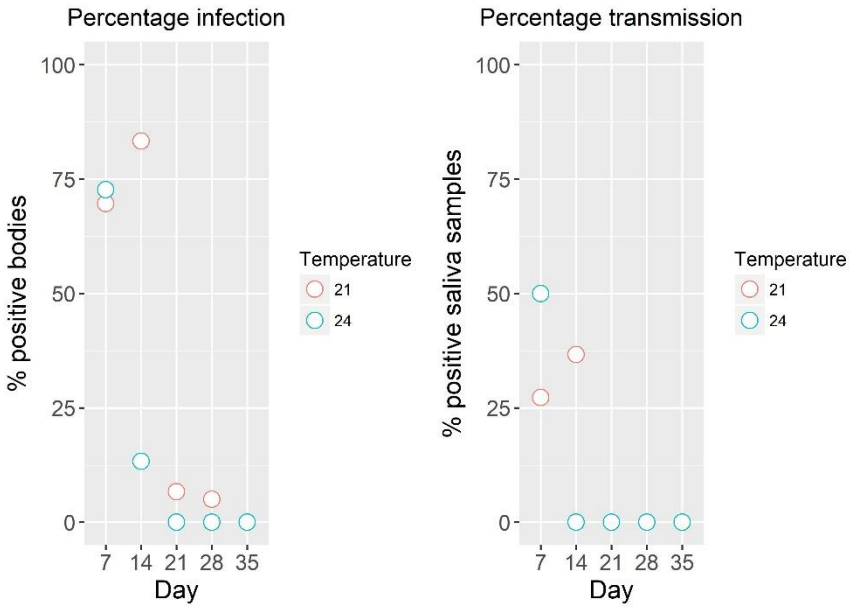

Figure 4.4 Proportion of infected *Oc. detritus* and those with the ability to transmit RRV per temperature and time point.

This observation correlates with the drop in estimated quantity of RNA detected in these bodies seen at 21 °C in Figure 4.5.

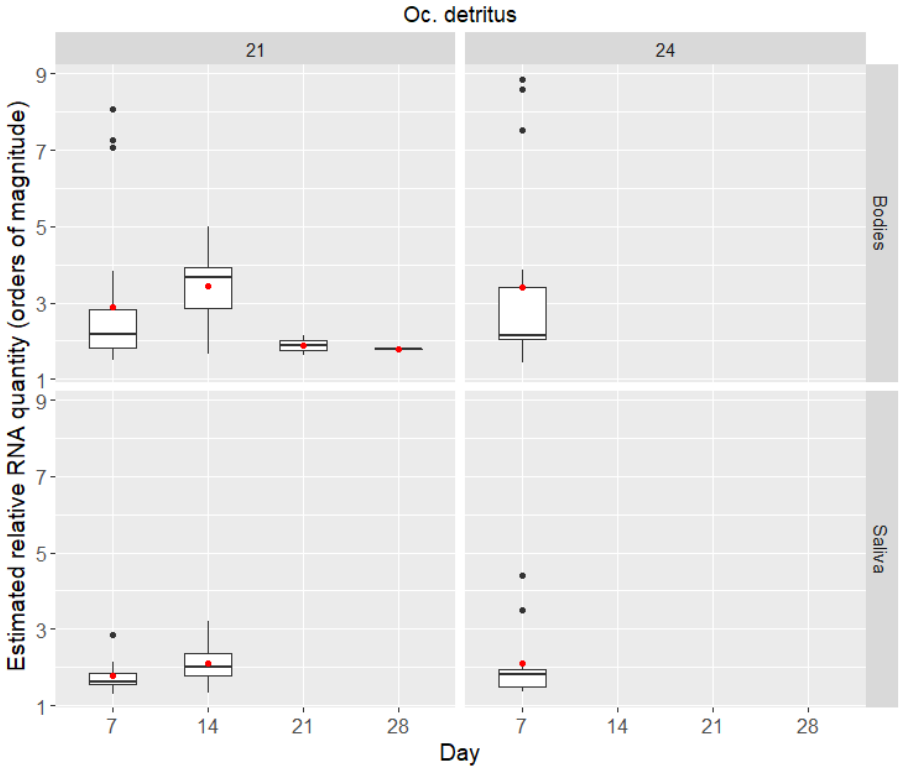

Figure 4.5 Range of estimated relative RRV RNA quantity in samples from *Oc. detritus* bodies and saliva, by time point and temperature (ºC).

Box and Whisker plots – boxes indicate 2nd and 3rd quartiles, vertical lines upper and lower quartiles, and horizontal lines the median. Black points indicate outliers. Red points indicate mean values.

CT values were not normally distributed (Shapiro-Wilks test) and variances were significantly different between groups. There were significant differences between incubation periods for CT values of mosquito bodies maintained at 21 °C (χ^2 =13.67, df = 3, P-value ≤ 0.01). Pairwise tests indicated a significant difference in CT values between mosquito bodies after a 14 day incubation period, compared to a 7 day incubation period at 21 °C (P-value ≤ 0.05). Although the trend shown in Figure 4.5 indicates a reduction in carcase estimated relative RNA quantities after 14 days, comparing values from bodies at other time points did not show a statistically significant difference. This is due to the small number of positive samples after greater than 14 days. There was also no significant difference between body CT values after a 7-day incubation, between mosquitoes incubated at 21 °C and those incubated at 24 °C. There was no significant difference in saliva CT values between 7 and 14 day incubation periods for mosquitoes maintained at 21 °C.

VECTOR COMPETENCE OF COLONY MOSQUITOES FOR RRV

Aedes albopictus challenged with RRV were incubated at 28 °C and tested at 12 (n = 34) and 14 days (n = 9). All 43 mosquitoes became infected with RRV and 42 of 43 (97.7%) were able to transmit the virus. The proportion of *Ae. albopictus* infected was significantly higher

than the proportion of *Oc. detritus* which became infected (P-value ≤0.01). The proportion of mosquitoes that could transmit the virus was also significantly higher for *Ae. albopictus* (P-value ≤0.001).

The median estimated relative RNA quantities in saliva at 14 days was approximately 5 orders of magnitude higher for *Ae. albopictus* than it was for *Oc. detritus*. Median relative viral RNA quantity in bodies was approximately 4 orders of magnitude higher in *Ae. albopictus* than it was for *Oc. detritus*, as illustrated in Figure 4.6.

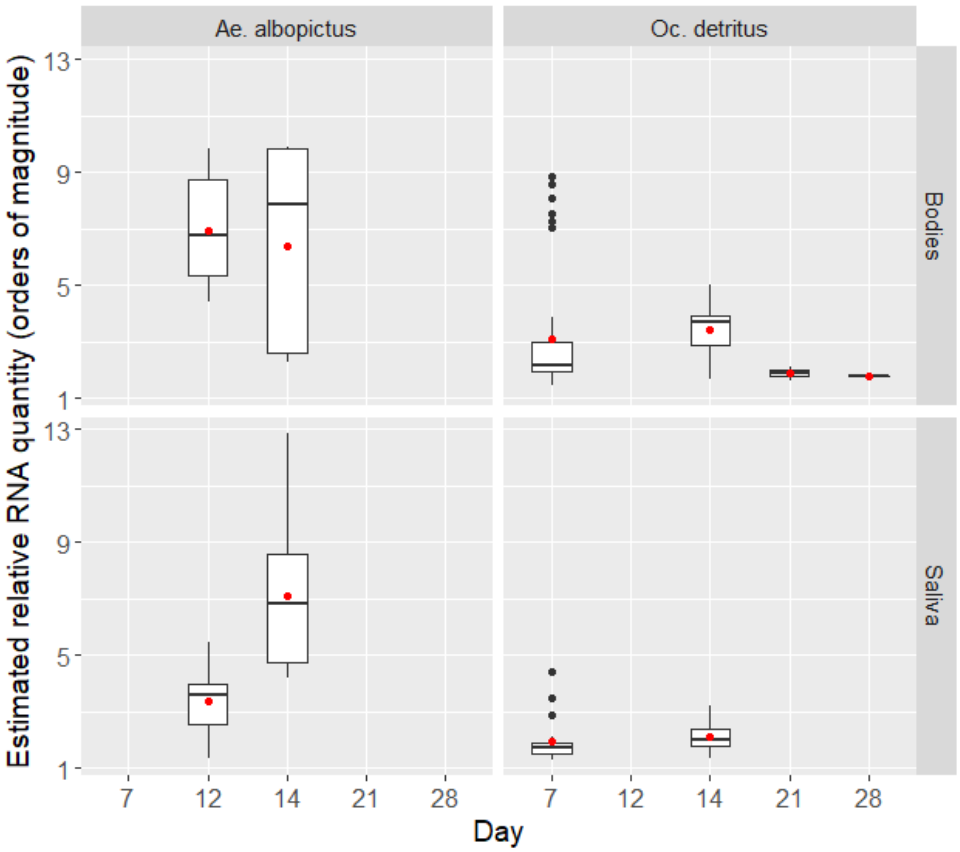

Figure 4.6 Estimated relative quantity of RRV RNA in samples from *Ae. albopictus* and *Oc. detritus* (all temperatures).

Box and Whisker plots – boxes indicate 2nd and 3rd quartiles, vertical lines upper and lower quartiles, and horizontal lines the median. Black points indicate outliers. Red points indicate mean values.

CT values obtained from *Ae. albopictus* saliva at 14 days were significantly lower (P-value ≤0.001) than those for *Oc. detritus* (Mann-Whitney U test). The differences in CT values between bodies of the two species at 14 days incubation were not significant (P-value = 0.234).

5.4.3 Venezuelan equine encephalitis virus

VECTOR COMPETENCE OF *OC. DETRITUS* FOR VEEV

After challenge with VEEV, *Oc. detritus* were maintained at 18 °C, 21 °C and 24 °C and sampled at 14, 21 and 28 days. Additionally, sampling after 7 days EIP for those mosquitoes maintained at 21 °C and 24 °C was undertaken. Infection data are shown in Table 4.7.

Temperature (°C)	Timepoint (days)	Number of mosquitoes in sample (n)	Number infected	Number transmitting	% infected	% transmission	Transmission rate (%)
18	14	25	3	0	12	0.0	0.0
	21	28	23	9	82.1	32.1	39.1
	28	30	23	2	76. 7	6.7	8.7
21	7	29	11	0	37.9	0.0	0.0
	14	30	23	3	76.7	10.0	13.0
	21	28	25	1	89.3	3.6	4.0
	28	15	5	0	33.3	0.0	0.0
24	7	30	22	7	73.3	23.3	31.8
	14	28	25	0	89.3	0.0	0.0
	21	27	27	6	100.0	22.2	22.2
	28	30	27	1	90.0	3.3	3.7

Table 4.7 Summary of infection and transmission rates of VEEV in *Oc. detritus.*

% transmission – proportion of mosquitoes tested, with VEEV in saliva. Transmission rate – proportion of susceptible mosquitoes transmitting VEEV.

In general, the trend is for increased proportions of mosquitoes infected Figure 4.7, and estimated relative RNA quantities over time (Figure 4.8), but few mosquitoes transmitted the virus.

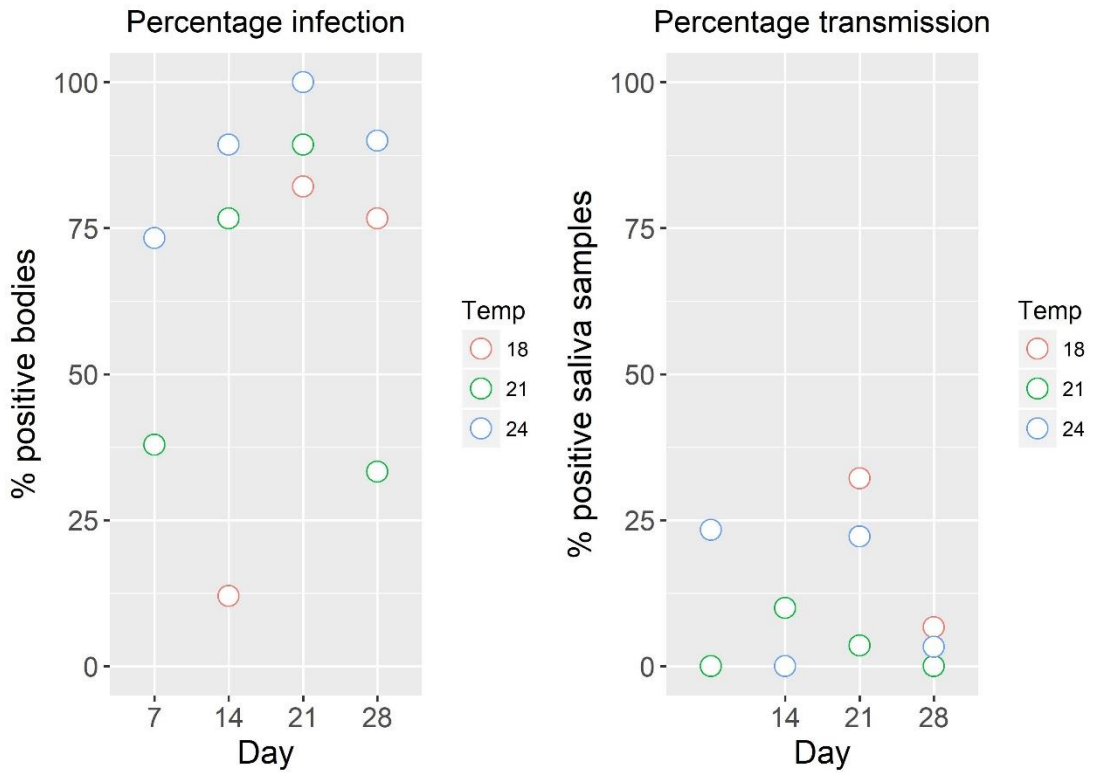

Figure 4.7 Proportion of infected *Oc. detritus* and those with the ability to transmit VEEV per temperature and time point.

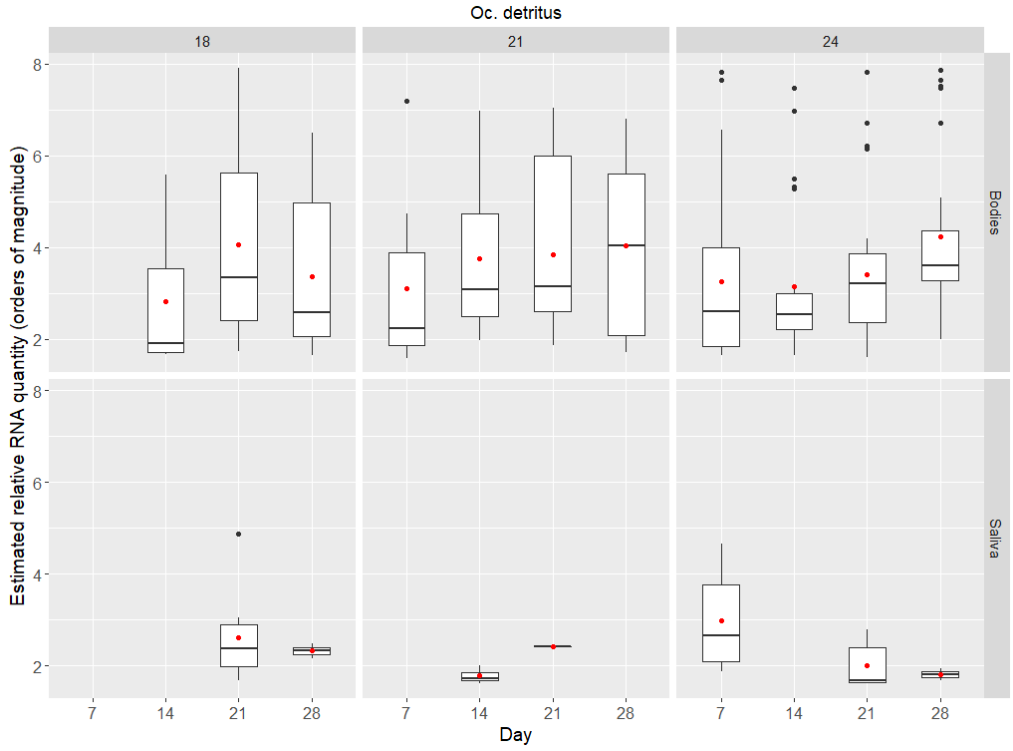

Figure 4.8 Range of estimated relative quantities of VEEV RNA in samples from *Oc. detritus* bodies and saliva, by time point and temperature (ºC).

Box and Whisker plots – boxes indicate 2nd and 3rd quartiles, vertical lines upper and lower quartiles, and horizontal lines the median. Black points indicate outliers. Red points indicate mean values.

VECTOR COMPETENCE OF *CS. ANNULATA* FOR VEEV

Cs. annulata were incubated at 24 °C after ingesting a blood-meal containing VEEV and were tested at 14, 21 and 28 days. Infection data are summarised in Table 4.8.

Timepoint (days)	Number of mosquitoes in sample (n)	Number infected	Number transmitting	% infected	% transmission	Transmission rate (%)
14	17	5	0	29.4	0.00	0.0
21	25	11	4	44.0	16.0	36.4
28	25	22	0	88.0	0.0	0.0

Table 4.8 Summary of infection and transmission rates of VEEV in *Cs. annulata*.

% transmission – proportion of mosquitoes tested, with VEEV in saliva. Transmission rate – proportion of susceptible mosquitoes transmitting VEEV.

Despite an increase infection rate over time to 88% at 28 days (Figure 4.9) and gradually increasing estimated quantities of viral RNA in bodies (Figure 4.10), only 4 out of 67 saliva samples were positive.

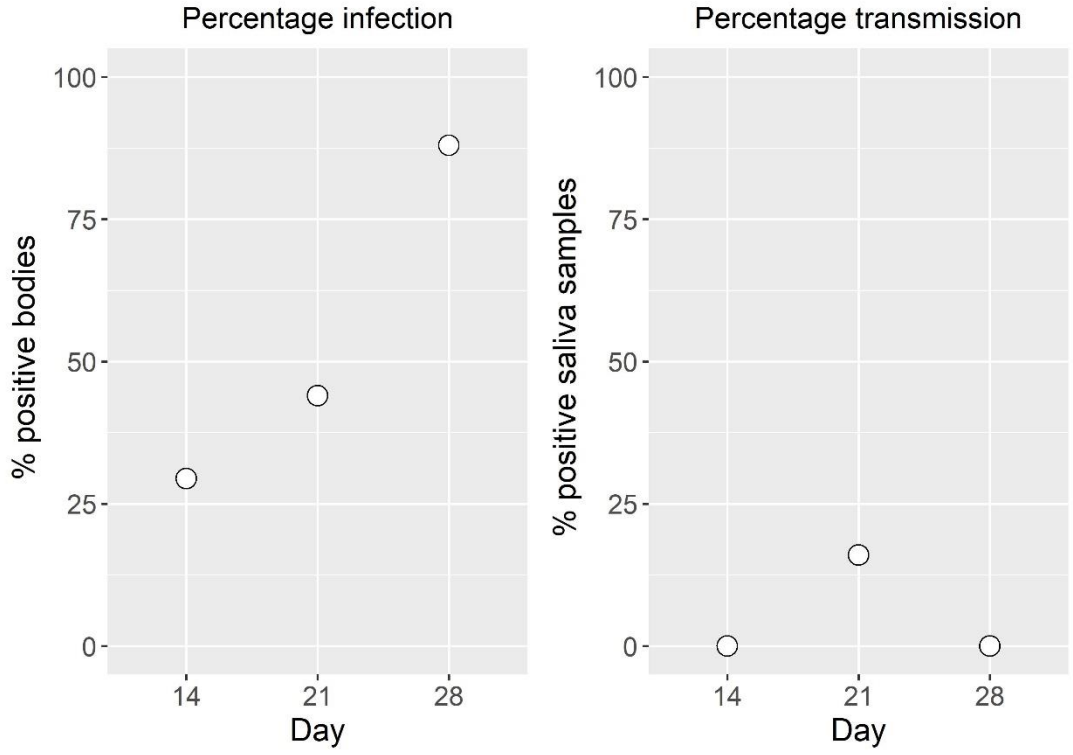

Figure 4.9 Proportion of infected *Cs. annulata* and those with the ability to transmit VEEV per time point.

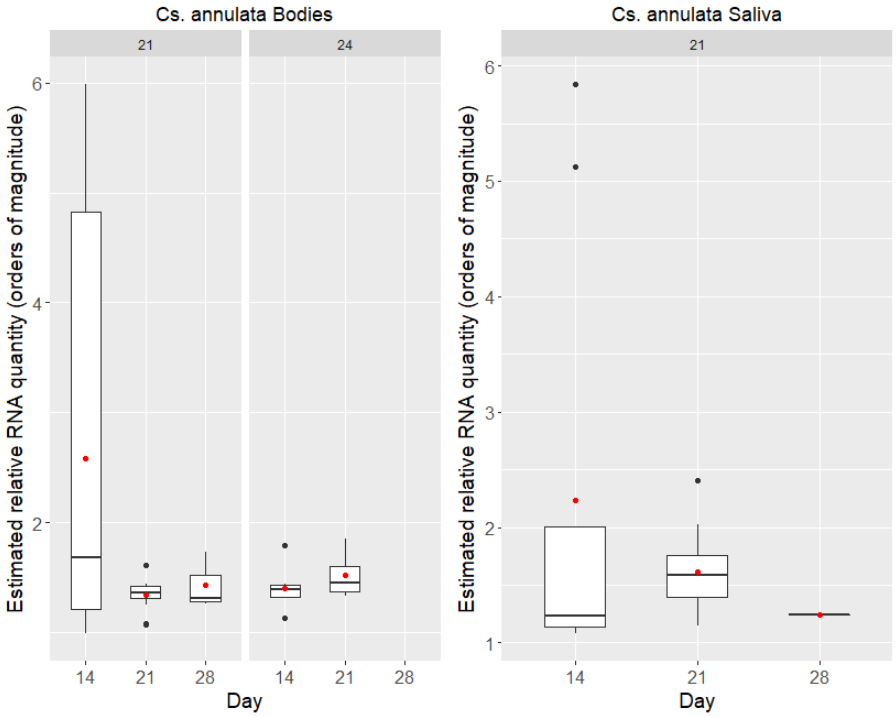

Figure 4.10 Range of estimated relative quantities of VEEV RNA in samples from *Cs. annulata* bodies and saliva.

Box and Whisker plots – boxes indicate 2nd and 3rd quartiles, vertical lines upper and lower quartiles, and horizontal lines the median. Black points indicate outliers. Red points indicate mean values.

VECTOR COMPETENCE OF COLONY MOSQUITOES FOR VEEV

Ae. albopictus were challenged with VEEV and incubated at 27 °C for 10 days (n=27) or 14 days (n=13). *Ae. aegypti* (n=3) were challenged and incubated at 27 °C for 14 days. None of these produced positive PCR results in carcases or saliva samples.

Discussion

In the present study, *Oc. detritus* was shown to be laboratory vector competent for RRV. Despite high proportions of mosquito carcases being infected, VEEV RNA was usually not demonstrated in the saliva of *Oc. detritus* making this species unlikely to be an efficient vector. *Cs. annulata* was demonstrated to be laboratory competent for JEV and unlikely to be an efficient vector of VEEV: most mosquito carcases were positive for viral RNA at 28 days, but few produced virus RNA in saliva. *Oc. punctor* was also shown to be laboratory vector competent for JEV. These results are the first demonstration of vector competence of any UK (European) mosquito for VEEV and RRV. Perhaps one of the most interesting results of the present study is the finding that *Cx. pipiens* is potentially an efficient vector of JEV at 18 °C, demonstrating 72.2% transmission (RNA in saliva) at 21 days.

A previous study has shown that *Cx. pipiens* is capable of laboratory transmission of JEV after 11 days when maintained at 27 °C (de Wispelaere et al., 2017). Lower incubation temperatures have not been previously tested for this mosquito-virus pair, to the author's knowledge. Further work to estimate the EIP of JEV in *Cx. pipiens* at low temperatures is warranted. *Cx. pipiens* is a widespread vector of West Nile virus (Andreadis et al., 2004, p. 200; Farajollahi et al., 2011; Turell et al., 2002) and it may prove necessary to re-assess the risk of JEV emergence in temperate regions where other ecological risk factors are present.

This is the first study of *Oc. detritus* demonstrating laboratory vector competence for an alphavirus. Previous studies with this species have also demonstrated laboratory competence of *Oc. detritus* for JEV and WNV, but not for chikungunya virus, an alphavirus (Blagrove et al., 2016).

The results for RRV and *Oc. detritus* have several interesting aspects. The maximum percentage potential transmission in this study was 50% at after 7 days at 24 °C. Infection, transmission and relative viral RNA all dropped after an initial peak and reached zero for all parameters by 21 days at 24 °C and by 35 days at 21 °C, suggesting viral clearance by *Oc. detritus.* Apparent virus clearance in an initially susceptible mosquito species is an unusual but not unknown phenomenon. *Cx. tarsalis* incubated at 32 °C appeared to eliminate viable WEEV whereas those incubated at 18 °C did not and those incubated at 24 °C showed decreased transmission rates at 12 days compared to 6 days (Hardy et al., 1983). Reduced transmission but not infection rates were also seen in *Cx. tarsalis* for WEEV at more realistic temperatures of 20 °C and 25 °C by (Reisen et al., 1993). Reduction in titre in bodies and salivary glands from 7 to 14 days incubation for RRV has been demonstrated in *Oc. vigilax* (Kay and Jennings, 2002) in adults maintained at 18 °C and 25 °C. The latter study also indicated that RRV produced higher titres in mosquitoes incubated at 18 °C and 25 °C than those maintained at 32 °C. Similar results were also shown for *Ae. albopictus* in another study (Nicholson et al., 2014). *Oc. vigilax* is a major coastal vector of RRV in Australia and lowered titres did not correspond with a significant decrease in the proportion of salivary gland infections over time (Kay and Jennings, 2002) and titre is considered not to be an important factor in transmission (Jennings and Kay, 1999). However, the decreasing titres over time and the lower titres at higher temperatures bear similarity to the results of the present study. The mechanism behind apparent virus clearance is not understood (Hardy et al., 1983; Kay and Jennings, 2002). And indeed true viral clearance by insects is considered not to occur (Cheng et al., 2016; Vodovar and Saleh, 2012). Insect immunity is not completely understood and one of the major mechanisms is RNA interference, but this has generally not been demonstrated to eliminate the virus, but rather to control it at tolerable levels (Cheng et al., 2016). To further investigate this phenomenon, experiments with RRV

and *Oc. detritus* should be repeated. For example, by using an incubation temperature of 24 °C and a combination of non-destructive sampling at 7 days to confirm infection (Fontaine et al., 2016; Hall-Mendelin et al., 2010) and final destructive sampling at 21 days to confirm or refute viral clearance (or clearance of viral RNA, as the hypothesis is that there will be none). Confirming viral clearance could include intracerebral inoculation into suckling mice (Mims et al., 1973) as very low copy numbers of alphaviruses can be detected this way (Smith et al., 2005), cell culture, or using techniques for identification of individual RNA copies (Cella et al., 2013), in PCR-negative samples.

Cs. annulata has only been tested once before for vector competence for an arbovirus (Danielova, 1972): it was shown to be competent, but was not an efficient vector of Tahnya virus (Bunyaviridae; Orthobunyavirus), taking between 52 and 86 days to reach an infection rate of 100%. However, the maximum blood meal titre used by Danielova was $10^{3.5}$ $SMLD_{50}$, as viraemic titres of only $10^{2.3\text{-}3.4}$ had been described in rabbits and hares, the amplifying hosts (Simková, 1963). The slow replication of Tahnya virus can be compared to the results obtained in the present study with VEEV infection, although Danielova simulated hibernation after 14 days by altering the incubation temperature to 10 °C. In an early study, a mosquito of the same genera, *Cs. inornata*, was shown to be laboratory competent for the Nakayama strain of JEV, however, the dose of virus used was not titrated (Reeves et al., 1946). In the present study, *Cs. annulata* were not tested at 7 days due to restrictions on the number of mosquitoes available. Only one maintenance temperature (24 °C) for assessment of VEEV competence was used for the same reason. *Cs. annulata* was an inefficient laboratory vector of VEEV and replication was slow, with a gradual increase in the percentage of infected mosquitoes (RNA detectable in bodies) from day 14 to day 28. For *Cs. annulata* and JEV, maximum percentage potential transmission (30%) occurred at 14 days after incubation at 21 °C. Reduced infection rate and potential transmission were seen by comparison in those incubated at 24 °C. In fact, there was no transmission (no RNA detectable in saliva) at any time point from samples incubated at 24 °C. As *Cs. annulata* were not tested after 7 days of incubation it is possible that a similar phenomenon to that described for *Oc. detritus* and RRV occurred. In fact, a decrease in transmission rates and gradual reduction in apparent infection rates has been described previously for SLEV (another flavivirus) at high temperatures, relative to the temperatures the vector is normally exposed to (Reeves et al., 1990). The mechanism behind these observations was not discussed in that study, although it may be possible that the unnaturally high incubation temperature for British *Cs. annulata* may affect the metabolism of the mosquito and reduce virus replication. Mosquitoes for these experiments were collected later in the season than those maintained at 21 °C. Variation in the vector competence of wild-caught mosquitoes

has also been shown throughout a season in a previous study on WEEV and *Culex tarsalis* (Reeves et al., 1990).

The results of this, and previous studies, demonstrate that temperature can have more complex influences on vector potential than a simple degree-day model with a linear relationship between temperature and infection rate (1/EIP) might suggest: for example lower holding temperatures than larval rearing temperatures can reduce infection rates for flaviviruses, the mechanism for which is not known (Kay et al., 1989). For VEEV it has been demonstrated that *Oc. taeniorhyncus* reared at 19 °C had higher infection rates than those reared at 26 °C, regardless of the adult holding temperature (19 °C or 26 °C) (Turell, 1993). Conditions in the present study may be similar, where larvae collected at the beginning of the season may have had different rearing conditions to those collected later in the season.

In this study, virus titres in blood meals were chosen to simulate those produced in viraemic hosts. This information is generally available only from experimental infections and therefore the host species tested may be limited, for example not all reservoir species for WNV or EEEV are likely to have been tested for levels of viraemia, as many reservoir hosts exist, and for EEEV, transmission cycles in some regions are not well understood. This type of experiment is expensive and technically challenging as hosts must be maintained in a Containment Level-3 or Biosafety Level-3 facility. Therefore, ecologically relevant hosts may exist which produce higher or lower levels of viraemia than those published. However, the main aim of the present study was to confirm or refute competence under the most likely favourable conditions for both virus and vector. Future work could include assessment of vector competence at lower virus titres than those used in this study.

Infection of colony mosquitoes as positive controls to confirm that the techniques used in this study could demonstrate transmission potential, were only moderately successful. Of the colony mosquitoes, *Ae. albopictus* was a highly efficient laboratory vector of RRV, with 97.7% with RNA in saliva, and produced high estimated relative RNA quantities in carcases and saliva. *Ae. albopictus*, *Ae. aegypti* and *Cx. quinquefasciatus* were all inefficient vectors of JEV in the present study, with a maximum percentage transmission (RNA in saliva) of 25% for *Ae. albopictus* and low estimated relative RNA quantities were produced in all cases. For VEEV, neither the *Ae. albopictus* nor the *Ae. aegypti* tested were susceptible to infection. The close to 100% infection and transmission (saliva positive for RNA) rates found for RRV in *Ae. albopictus* demonstrated that consistent results were achieved during this study. This high rate of transmission contrasts with a previous study in which only 25% transmission in *Ae. albopictus* was reported (Nicholson et al., 2014). In that study blood meal virus concentrations of approximately 10^4 $TCID_{50}$ were used, in comparison to the

maximum 5.6 x 10^6 used in the present study. Australian native hosts of RRV have been shown to produce viraemic titres of 10^7 (Boyd et al., 2001; Ryan et al., 1997) when infected by mosquito bite. For JEV, 25% transmission was demonstrated with *Ae. albopictus*, but with low levels of viral RNA in saliva and carcases compared to those for *Cx. pipiens*. Another study (de Wispelaere et al., 2017) demonstrated 63% transmission with another g5 strain of JEV in *Ae. albopictus* using a higher dose of virus. Transmission of JEV by *Cx. quinquefasciatus*, in the present study, was only 3.3%, compared to 29% reported previously by colleagues for a different strain of JEV (MacKenzie-Impoinvil et al., 2014). The mosquito strain used in that study (RECIFE) was not available and a strain from Tanzania (MUHEZA) was the only available alternative. Vector competence (or lack thereof) of individual strains of the globally distributed species *Cx. quinquefasciatus*, for JEV, is highly variable (Huang et al., 2015; van den Hurk et al., 2003). Low levels of infection and transmission of JEV in *Ae. aegypti* were as expected, as JEV is thought to be controlled by *Ae. aegypti* through RNA interference (Sasaki et al., 2017), although at least one study has shown both infection and salivary gland infection (van den Hurk et al., 2003). The lack of any positive results for colony mosquitoes challenged with VEEV was somewhat unexpected, but again could perhaps be explained by strain variation. A previous study (Smith et al., 2005) used a different epizootic strain of VEEV and *Ae. albopictus* sourced originally in Texas and achieved 57% infection rates using $10^{6.1}$pfu/ml in artificial blood meals, compared to the 0% using $10^{6.9}$ (9.5 x 10^6) used in this study. The primary purpose of the positive controls was to confirm that the experimental procedure could detect viral RNA, in the event that UK mosquito species results were negative, thereby confirming their lack of vector competence. As UK mosquito species were all capable of producing detectable RNA in saliva, this function was no longer critical. The second purpose was to be able to compare the levels of transmission in UK species with those of established vectors. This function was only fulfilled for RRV with *Ae. albopictus.* Thirdly, colony mosquito and virus strain pairs which had not been tested previously, were investigated.

Whilst vector competence of local mosquitoes is crucial information in assessing the risk of outbreak occurrence in the event of virus introduction into a region, it should be noted that the complex nature of the ecology and epidemiology of mosquito-borne equine arboviruses makes risk prediction very challenging. Certainly, policymakers should be aware of the likelihood of increasing risk in future, and at the very least monitor the situation in southern Europe as a potential early warning of increased risk of arbovirus transmission.

For the viruses used complete passage information was not available. VEEV and RRV were known to have been passaged in Vero cells in the present study and were believed to have been passaged in mouse brain, but passage numbers were unknown. Sequencing of virus

genomes was not undertaken due to financial constraints. Therefore, the possibility that adaptation to mammalian cells may have altered the ability of these viruses to infect mosquitoes cannot be discounted (Smith et al., 2005). To minimise this, low passage number strains derived recently from the field may be available for some viruses, but, to avoid adaptation to cell culture, the ability to create infectious cDNA clones from published sequences and produce infectious virus is required. This was beyond the scope of the present study. Tissue culture methods of titre assay such as plaque assay would be ideally used for future work to confirm blood meal virus titre and could be used to confirm both negative and positive saliva results, to discount problems in RNA extraction, for example.

In conclusion, the present study has demonstrated that mosquito species present on UK equine premises can transmit equine arboviruses. *Oc. detritus* was demonstrated to be a laboratory competent vector for RRV and an inefficient laboratory vector for VEEV. *Oc. punctor, Cx. annulata* and *Cx. pipiens* were all demonstrated to be laboratory competent vectors of JEV. Further work on the EIP of JEV in *Cx. pipiens* should be considered a priority for European risk assessments.

Appendix to Chapter 4

To aid the interpretation of CT values on plots, CT values are converted to 'estimated relative RNA quantity' compared to a sample with a CT value of 40 for each viral RNA. This is then presented on a $\log_{10}$ scale where a CT value of 40 was represented by a log value of approximately 1.

Although this method of presentation produces a scale similar to that used for viral titres, it is important to remember that this is not what is being reported: PCR results could be described as semi-quantitative as the method does not fulfil requirements for absolute quantification of viral RNA. Absolute quantification of viral RNA was beyond the scope of this study, and requires rigorous quality control of RNA samples, quantification calibrators such as synthetic RNA or other recognized standards (Bustin et al., 2009) and ideally, internal controls to monitor for reaction inhibitors (Schwaiger and Cassinotti, 2003).

A standard curve for the PCR was generated using 3 replicates of 10-fold serial dilutions with a dynamic range of 7 logs using the stock virus. The average CT values produced from this qPCR run were plotted in Microsoft Excel to produce an equation and correlation coefficient (R^2) for the best fit line using Least Squares estimation (Figure 4.11)

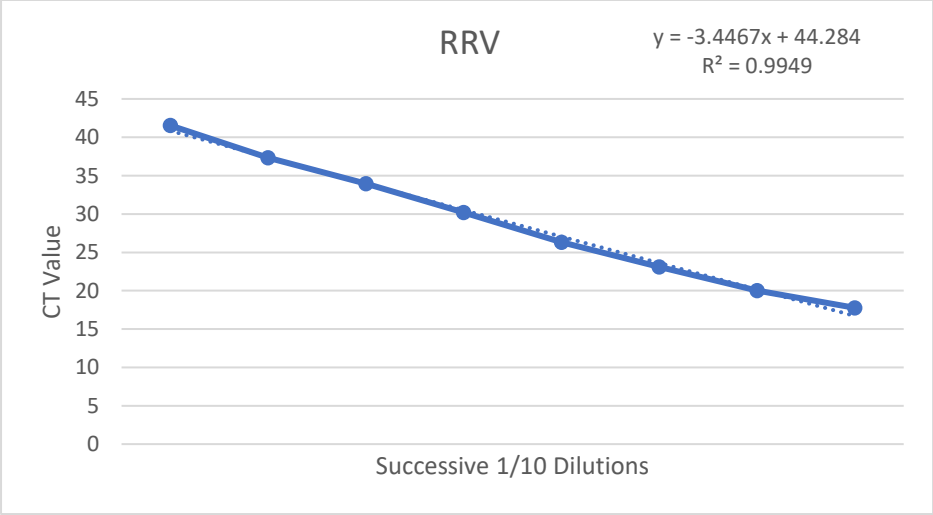

Figure 4.11 Standard curve plot for RRV, including the equation for best-fit line and R-squared value.

Where:

$$y = mx + b$$

For RRV, then:

slope (m) = -3.4467

intercept (b) = 44.284

Efficiency of the PCR reaction is calculated thus:

$$Efficiency = -1 + 10^{(-\frac{1}{slope})}$$

For RRV, Efficiency = 95.04%

For quantitative PCR the formula used to calculate copy number, is:

$$X_0 = E_{AMP}^{(b - Cq)}$$

Where:

The standard curve equation (1) gives b and m

X_0 = copy number

Here, 'estimated relative RNA quantity' has been used instead of RNA copy number:

$$\text{Estimated relative RNA quantity} = E_{AMP}^{(b - Cq)}$$

Where:

E_{AMP} = exponential amplification value (Table 3.2) = $10^{(-1/m)}$

Cq = arithmetic mean of technical replicate CT values for each sample (Cq = quoted CT values in this text).

Therefore, for RRV:

$$E_{AMP} = 10^{(-\frac{1}{-3.4467})} = 1.95$$

And:

$$\text{Estimated relative RNA quantity } (X_0) = \log_{10} 1.95^{(44.284 - Cq)}$$

So for RRV, a CT value of 40 corresponds to 1.24 on the log10 scale, and a CT value of 20 corresponds to a value of 7.04.

It can therefore be seen that this method of presentation is based on the same scale as might be used to present viral RNA copy number, but results presented in this study are an estimate of RNA quantity relative to the other samples.

5 FIELD-TESTING OF REPELLENTS FOR THE PROTECTION OF HORSES FROM MOSQUITO BITING

Abstract

It is important that veterinary surgeons and owners are equipped with evidence-based protection methods for individual horses, in areas with a risk of arbovirus transmission. For some arboviruses affecting equines, there are no vaccines available and spray repellents are commonly used by horse owners to deter biting flies. There are no peer-reviewed studies of the efficacy of formulated repellent products on the market for the protection of horses from mosquitoes, and few studies for technical grade repellents with respect to horses and mosquitoes. Therefore, a field trial of two products marketed in the UK and commonly used by horse owners for protection of equines from biting insects was conducted. Products were NAF Off® DEET Power Spray (19.7% DEET) and NAF Off® Citronella Spray. Natural mosquito exposure was used to test repellents by sampling mosquitoes from two horse baited net traps using an untreated (control) and a repellent-treated horse. Two pairs of horses were used for each spray product. The percentage repellency of NAF Off® DEET Power Spray was estimated to be 95.3%, meaning that for every 100 mosquitoes which bite an untreated horse, 4.7 are expected to bite a horse treated with this product. The efficacy of Naff Off® Citronella Spray as a mosquito repellent was demonstrated to be poor. The method described here has been shown to be practicable and could be used in further work required to confirm if there is a place for repellent products in the protection of individual horses from arboviral disease in the event of heightened regional risk: i.e. if complete protection (100% repellency) can be achieved for an adequate time period using alternative products. However, it seems unlikely that repellent use can guarantee complete prevention of mosquito biting, therefore vaccines are the most appropriate prevention method against arbovirus infection. These results show that DEET- containing products have a place in the reduction of nuisance biting of horses by mosquitoes and suggest that there may be some benefit in their use in circumstances of low to moderate transmission risk of arboviral disease, in combination with other measures to mitigate biting rates, where vaccination is not possible.

Introduction

In the event of autochthonous transmission of equine arboviral disease in the UK, reduction in blood-feeding by mosquito vectors on horses has the potential to reduce the risk of arboviral infection of the individual horse. General control methods to prevent horses being bitten by mosquitoes include the topical or environmental application of insecticides, repellents, insect-proof barriers in stables, physical barriers such as fly rugs and mosquito population control methods. The latter include removal of habitats that provide breeding sites, such as stagnant water and biological control methods such as the 'biological pesticide' *Bacillus thuringiensis israelensis.*

Given that 22-44% (Harris, 1999; Hotchkiss et al., 2007) of horses are kept on premises that do not belong to the horse owner (ie, livery yards, farms or riding schools), it may be relatively difficult for concerned owners to implement protective measures that involve changing the environment. Individual, horse-level measures of protection, such as repellents, may be preferred and faster to implement. In addition, in the UK, the majority of horses are turned out at pasture for significant periods of time during the summer months. In one study of 873 horse owners, the median time at pasture during the summer was 23 hours per day (Hotchkiss et al., 2007). Also, the potential mosquito vectors of arboviral disease in the UK are mainly exophilic (bite outdoors), therefore bite protection methods effective when horses are at pasture are desirable.

Potential protection methods for individual horses from mosquito bites at pasture include spray repellent products, fly rugs and masks and permethrin impregnated rugs. In the survey of UK horse owners conducted for this thesis (Chapter 3) the majority of respondents (90.1%) indicated that they used repellents on their horse(s) in order to reduce insect biting nuisance. Repellents are not considered medicinal under UK or EU legislation and therefore do not require licensing under veterinary medicines legislation. Efficacy does not, therefore, have to be tested in the species for which the product is marketed. There have been no peer-reviewed, published field studies specifically testing the efficacy of formulated repellent products for the protection of horses from mosquitoes, to the author's knowledge.

The purpose of this study was to investigate the efficacy against mosquitoes of two formulated products that are marketed in the UK and are commonly used by horse owners for prevention of biting by insects, including mosquitoes. Evidence-based information about the efficacy (or lack of) of such repellents will be of critical importance, in the event of heightened risk of or an outbreak of an arboviral disease in horses in the UK, in minimising the development and spread of disease.

Materials and Methods

6.3.1 Site and Animals

The site chosen for this field experiment was site 8 (approximate location: Latitude 53.3, Longitude 3.0) in the previously published mosquito sampling paper (Chapman et al., 2016) and in Chapter 2. This site is situated on the Wirral Peninsula, close to the Dee estuary and was shown during previous work to have a high vector density and variety of mosquito species. This site was considered suitable since many horses and ponies owned by the same person were housed at this site and were used for commercial purposes. Based on results obtained in Chapter 2, site 7 had a greater vector density than site 8, likely due to its close proximity to large areas of marsh suitable for *Ochlerotatus detritus*. However, the horses at site 7 were pleasure and competition horses owned by private individuals and it was considered unrealistic that these could be used in the experiments described.

Horses were recruited in pairs and were matched as closely as possible for size. The selection criteria used for recruitment were that the horses were healthy (with no history of a skin condition) and did not have a history of being treated with any topical insecticide within the last two months and no repellent or worming treatment within the previous month. Also, the horses must not have normally worn a fly rug, as this could affect the distribution of applied repellent, and any change in the normal management of the horses due to the experiments (other than the application of the repellents) would have meant that a Home Office licence was required. Horses were owned by the same individual and ranged in age from 6 years to 15 years.

Ethical permission for this study was obtained from The University of Liverpool Veterinary Ethics Committee (VREC444).

6.3.2 Experimental setting

Experiments took place in October 2016. The study period was based upon a high forecasted abundance of adult *Oc. detritus.* Peak seasonal density of this nuisance biting mosquito in the area was considered necessary to obtain adequate data from the experiments described. Monitoring of the Dee marshes for the collection of larvae and pupae for laboratory experiments (Chapters 4 and 6) was ongoing throughout the season from late April to October and therefore peaks in adult density could be predicted (Clarkson et al., 2016). During late August and early September, larval stages of *Oc. detritus* were obtained for laboratory experiments. However, the tidal pools that the larvae had been obtained from dried up before adult emergence could occur. These pools are filled by high tides and water levels can be

maintained by precipitation between high tides. Significant numbers of pupae were observed towards the end of September and therefore increased biting nuisance was expected at the beginning of October.

Two galvanised steel cages used for a previous horse study (Robin et al., 2015) were used. Each cage was 2.4m long x 1.2m high x 2.4m high. Modifications were made to these cages, to allow for the rear of the cage to be opened, and extensions were fitted to the tops of the cages so that there was space between the cage walls and the net for a collector to sample mosquitoes resting on the net. The front of the cage itself was open, apart from a webbing restraint at chest height, to allow for rapid and safe exit from the cage if the horse became distressed. The two cages were situated approximately 12 metres apart, to avoid repellents influencing mosquitoes captured on the respective control horse but to minimise the effects of different locations. Both cages were securely fixed to the ground using eight large marquee stakes so that there would be no risk of entrapment if the horses kicked out at the cage. The cages were covered with netting of an aperture size $<1.5mm^2$ so that blood-fed mosquitoes would rest on the inside of the net. A space of 10cm was left at the bottom of the netting so that mosquitoes could enter the cage (Figure 5.1). The netting at the front of the cage was left open to a height of 1 m (above head height for the horses used) whilst the horse was in the cage so that the horse was not completely enclosed and to comply with advice regarding non-requirement of a Home Office licence for this experiment. Once the horse was removed from the cage at the end of the trapping period, the front of the net was immediately dropped to trap mosquitoes inside the net.

Figure 5.1 Cages and nets during the set-up process.

All horses were trained to walk into the cages before trapping sessions were commenced, by walking them through the cage with the net in place, several times.

6.3.3 Pilot Work

In the days immediately before trapping sessions with repellents took place, the nets and the predicted density of biting mosquitoes were tested, and the predicted peak time of biting was investigated. One horse was used, with no repellent applied, for 4 hours before sunset on two subsequent days, to collect blood-fed mosquitoes from inside the nets. It was apparent that peak biting rate occurred in the hour before and immediately after the forecast sunset time.

Therefore, subsequent trapping took place for 90 minutes before the forecast sunset time (Her Majesty's Nautical Almanac Office, n.d.) and for 30 minutes afterwards (before the end of civil twilight). This is the peak biting time for *Oc. detritus* (Service, 1971b).

6.3.4 Experimental Design

The experimental design was based on a replicated latin square design with 2 'blocking factors' that attempted to controlling for two sources of nuisance variability. In this case, the potential causes of nuisance variability were 'day', 'horse' and 'cage' (location). It was considered that because the cages were only 12 metres apart and cages and nets were identical it was likely that 'cage' would create minimal variability. 'Horse' and 'cage' were combined into the same blocking factor by always keeping each individual horse of a pair in

the same cage for all experiments, in order make the experiment practical with 2 cages, a limited time scale (based on the peak of mosquito density), and to minimise degrees of freedom for analysis. Residual effects of repellents on the cages seemed unlikely as the repellents had been dried onto the coat several hours before horses entered the cages. Two readily available spray products commonly used by horse owners were used as treatments for testing (Chapter 3). These were NAF Off ® DEET Power Spray (NAF, Monmouth) containing 19.6% DEET (N,N-Diethyl-m-toluamide) and NAF Off ® Citronella Spray (NAF, Monmouth) containing an undisclosed quantity of citronella oil. A wash-out period of 7 days was used after repellent application before a horse could be used as a control.

REPELLENT APPLICATION

NAF Off ® DEET Power Spray or NAF Off ® Citronella Spray was applied to test horses 7 hours before the forecast sunset time. This time point was chosen to provide a realistic time of protection required to make repellents of practical use for the grazing horses in the UK. A sponge was used to apply repellent to the entire hair coat and to ensure that it was damp. This protocol for repellent application was based on protocols used commercially for testing repellents for use on horses (Donahue, 2009). 200-250 ml of repellent was used depending on the size of horse and type of hair coat. In general, longer or finer haircoats required a larger volume of repellent to ensure adequate coverage and larger horses required a larger volume for similar hair coat types. If the test horse was turned out in the rain or had a rug put on during the day of the test, the test was abandoned.

6.3.5 Collection and storage of mosquitoes

During the trapping period, a mechanical pooter (Watkins and Doncaster, Leominster, UK) was used to sample all mosquitoes resting on the inside of the net. This was performed every ten minutes throughout the trapping period. At the end of the trapping period, the horses were removed from the cages and the front netting was dropped. Two collectors simultaneously collected the mosquitoes that were then left inside the nets.

At the end of the collection period, mosquitoes were immobilised with ‘Fly-nap’ (Carolina Biological Supply Company, Burlington, NC, USA). They were then transported immediately to the laboratory and were stored in 1.5ml Eppendorf tubes in 70% ethanol at -20 °C (within 2.5 hours of collection).

6.3.6 Blood meal analysis and individual horse discrimination

Mosquitoes were examined whole under a microscope to identify if they were blood-fed and samples were labelled accordingly. To obtain samples to act as a positive control both for PCR amplification and for the individual horse, one mosquito was sampled from each horse whilst taking a blood meal. Additionally, a hair sample including the hair bulb was obtained from each horse.

All blood meals from mosquitoes collected from nets containing repellent treated horses were analysed to confirm that the blood-meal was obtained from the individual bait horse in the net from which the mosquito was collected had been obtained from a horse. No mosquito bites were recorded on the human collectors during these experiments. Due to financial constraints, only a proportion of mosquitoes from control nets could be analysed to confirm the blood-meal source. A sample size calculation was performed to identify the number of mosquitos (60) that should be tested from control nets to give a confidence level of 0.95 with an expected proportion of 95%, (having a blood meal from the expected bait horse) and a precision of 0.05 (Sergeant, 2017a).

MOLECULAR IDENTIFICATION OF MOSQUITO HORSE BLOOD-MEALS

DNA EXTRACTION

DNA extraction from mosquito blood meals was achieved using nexttec™ 1-step Tissue & Cells cleanPlates96 (nexttec™ Biotechnologie GmbH, Hilgertshausen, Germany).

Mosquitoes were removed from the storage vials and placed on absorbent paper to allow the ethanol to evaporate. 14 µl of Buffer G and 10 µl of Proteinase K were added to each well of a deep well plate. An individual whole mosquito was transferred to each well and homogenised using a micropestle. Samples were incubated at 56 °C in (with shaking) for 90 minutes. Meanwhile, 350 µl of Prep solution was added to each well of a nettec™ cleanPlate. The cleanPlate was incubated at room temperature for 5 minutes and then centrifuged at 350 x g for 1 minute. 100 µl of lysate was then transferred onto the cleanPlate and this was incubated at room temperature for 3 minutes. The cleanPlate was then centrifuged at 700 x g for 1 minute and the eluted sample was collected in a PCR plate. Samples were then stored at 4 °C.

PCR AMPLIFICATION

Primers, PCR amplifications and restriction enzyme digestion were as previously published (Millard et al., 2013). Two primer pairs were used to amplify two loci within the hypervariable D-loop of the mitochondrial control region:

D-loop, 232bp: AGGACTATCAAGGAAGAAGCTCTA and GTACATGCTTATTATTCATGGGGCA

D-loop, 397bp: AACGTTTCCTCCCAAGGACT and GTAGTTGGGAGGGTTGCTGA

Prior to use, these primer pairs were analysed using Primer-BLAST for specificity for the domestic horse *Equus caballus*. The first primer pair was specific to equids and the second primer pair produced Primer-BLAST results for only *Equus caballus* and *Equus przewalsii*. All positive samples were assumed to come from *Equus caballus*, the only horse species in the vicinity.

PCR amplifications for both fragments were carried out in 25 µl reactions with final concentrations of 1x Green PCR buffer, 2.0 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 mM of each primer and 1 unit of Platinum™ *Taq* Green Hot Start DNA Polymerase (Invitrogen). The PCR reaction was performed under the following conditions: an initial denaturation step of 94 °C for 3 minutes, followed by 40 cycles of 94 °C for 40 seconds, 55 °C for 40 seconds, and 72 °C for 1 minute, and ending with a final extension at 72 °C for 10 minutes.

RESTRICTION ENZYME DIGESTION

The restriction enzymes SssI (AciI) and Tru1I (MseI) were used to produce a restriction fragment length polymorphism (RFLP) banding pattern of the D-loop 232 bp fragment (Ishida et al., 1994) and the restriction enzymes SssI and TasI (MluCI) were used to produce a banding pattern of the D-loop 397bp fragment (Bowling et al., 1997). SssI recognizes CCGC(-3/-1)^ sites, Tru1I recognises TTAA (-3/-1)^ sites and TasI recognizes ^AATT sites. For SssI O buffer was used, for Tru1I R buffer was used and for TasI B buffer was used.

The 232 bp fragment was reported to have two possible cleavage patterns for Tru1I and three for SssI (Ishida et al., 1994).

- Tru1I
    - Morph A: 2 fragments (173 bp, 59 bp).
    - Morph B: 3 fragments (139 bp, 59 bp, 34 bp).
- SssI
    - Morph A: 1 undigested fragment (232 bp).
    - Morph B: shows heteroplasmy - 2 fragments and one undigested fragment (232 bp, 133 bp, 99 bp).

    - Morph C: shows heteroplasmy - 2 fragments and one undigested fragment (232 bp, 152 bp, 80 bp).

The 397 bp product was reported to have 3 possible cleavage patterns for SssI and two for TasI (Millard et al., 2013).

- SssI
    - Morph A: 1 undigested fragment (397 bp).
    - Morph B: 2 fragments (283 bp, 114 bp).
    - Morph C: 3 fragments (117 bp, 114 bp, 107 bp).
- TasI
    - Morph A: 4 fragments (181 bp, 105 bp, 66 bp, 45 bp).
    - Morph B: 3 fragments (181 bp, 150 bp, 66 bp).

For each digestion 12 µl of PCR product was used. To this was added 1.4 µl of the appropriate buffer and 1 unit of enzyme. The sample was mixed gently by tapping and briefly centrifuged. Samples were then incubated for 16 hours. Incubation took place at 37 °C for SssI digestion and at 65 °C for Tru1I and TasI digestion.

Results were visualised in 2% agarose gel after 115 minutes electrophoresis at 120V in 0.5x TAE (Tris-acetate-EDTA) buffer using peqGREEN (VWR Peqlab) staining at 1:25 000, and Gene ruler Ultra Low range Ladder (Thermo Fisher Scientific) was used to compare fragment sizes.

6.3.7 Data analysis

Percentage repellency was calculated using the following formula (Abbott, 1925):

$$\%\, repellency = 100\, x \frac{(C - T)}{C}$$

Where C = number of blood-fed mosquitoes trapped in control net

T = number of blood-fed mosquitoes trapped in treatment net.

Confidence intervals for a proportion were calculated using the Wilson method (Sergeant, 2017b).

Percentage repellency is the standard used in field testing formulated repellents for protection of human beings from mosquito bites (WHO, 2009), in which case mosquitoes are collected whilst attempting to feed, so feeding success is not recorded.

In order to gain as much information as possible about the effects of the treatments on mosquito behaviour 3 aspects of the data set were examined:

- Total number of mosquitoes captured from the net
- Number of blood-fed mosquitoes captured
- The proportion of blood-fed to non-blood-fed (feeding success)

Generalised linear models were used to analyse the outcome variables listed above, in R (R Core Team, 2017) and using the package MASS (Ripley and Venables, 2002).

Results

6.4.1 Mosquito Sampling

During the two pilot trapping nights (1 untreated horse, for 4 hours) 205 and 93 mosquitoes were captured and 198 and 70 mosquitoes, respectively, were identified as blood fed.

A total of 515 mosquitoes were sampled during 8 nights of trapping and 321 of these were identified visually as blood-fed (Table 5.1, Figure 5.2). Of the blood-fed mosquitoes, 314 were identified morphologically (Chapter 2) as *Oc.detritus,* 6 as *Cs. annulata* and 1 as *An. claviger*. Non-fed mosquitoes were identified as 134 *Oc. detritus,* 51 *Cs. annulata,* 6 *An. claviger,* and 2 *Culex (pipiens/torrentium).*

Sample	Treatment	Horse Number	Night	Cage	Total number of mosquitoes	Number of blood-fed mosquitoes	Number of unfed mosquitoes
D1R	DEET	1	1	1	6	6	0
D1C	Control	2	1	2	26	16	10
D2R	DEET	5	2	1	14	2	12
D2C	Control	6	2	2	23	12	11
D3R	DEET	2	5	2	19	0	19
D3C	Control	1	5	1	19	2	17
D4R	DEET	6	6	2	4	0	4
D4C	Control	5	6	1	46	24	22
C1R	Citronella	3	3	2	45	41	4
CIC	Control	4	3	1	36	24	12
C2R	Citronella	7	4	2	26	18	8
C2C	Control	8	4	1	59	42	17
C3R	Citronella	4	7	1	19	8	11
C3C	Control	3	7	2	55	35	20
C4R	Citronella	8	8	1	46	43	3
C4C	Control	7	8	2	72	48	24

Table 5.1 Total number of mosquitoes and number of blood-fed mosquitoes in each sample.

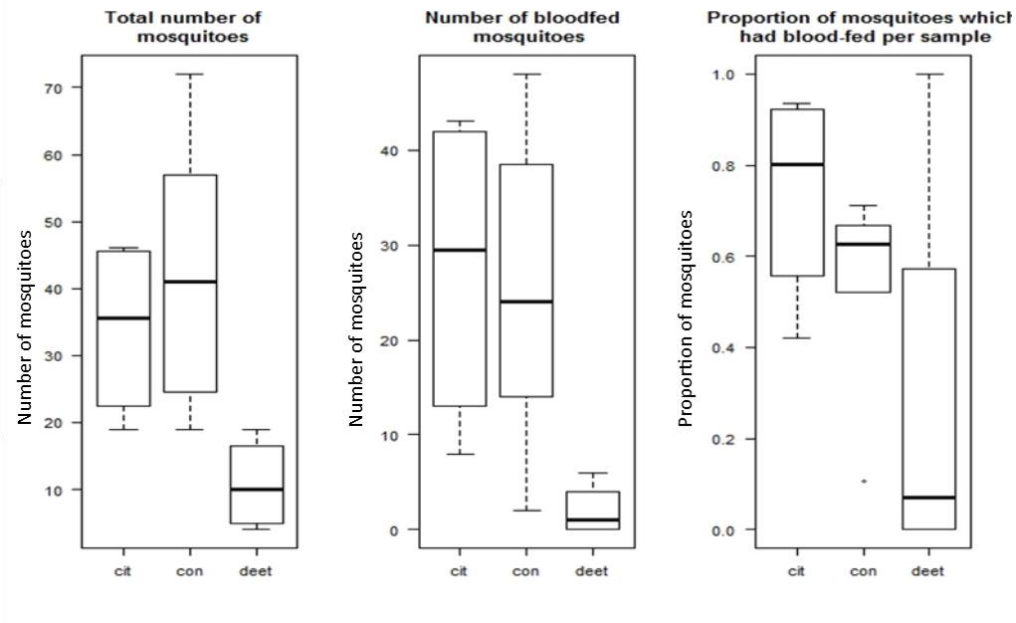

Figure 5.2 Boxplots of numbers of mosquitoes sampled and proportions of blood-fed mosquitoes.
cit – Naf Off® Citronella Spray, con – control condition, deet – Naf Off® DEET Power Spray

PERCENTAGE REPELLENCY

For DEET experiments a total of 8 blood-fed mosquitoes were trapped in treatment nets (i.e. apparently fed on DEET-treated horses), and 54 mosquitoes were trapped in control nets, giving a percentage repellency of 85.2% (95%CI; 73.4 – 92.3%) for NAF Off® DEET Power spray.

For Citronella experiments, a total of 110 blood-fed mosquitoes were trapped in treatment nets (i.e. apparently fed on Citronella treated horses), and 149 mosquitoes were trapped in control nets, giving a percentage repellency of 26.2% (95%CI; 19.8 – 33.8%) for NAF Off® Citronella spray.

6.4.2 Blood meal analysis

PCR products identified by gel electrophoresis were obtained for all blood meals analysed. Therefore, all blood meals were identified as having been derived from a horse. It was of interest to note that in the case of 4 mosquitoes that had been identified as partially blood fed, very little blood was noticeable at the time of homogenization. However, these samples also produced positive PCR results, indicating that rapid killing and storage of mosquitoes maintained equine mitochondrial DNA integrity in the blood meal. Two negative controls derived from laboratory reared (non-blood-fed) *Oc. detritus* did not produce positive PCR results.

The RFLP identification of individual horses did not definitively discriminate between all 8 horses in the study. It did, however, demonstrate that not all blood-fed mosquitoes sampled from each net had taken a blood meal from the expected horse. For the 8 horses, there were only 3 distinct RFLP patterns with 6 of the horses (numbers 2, 3, 4, 5, 6, 8) showing the same pattern (Table 5.2).

Horse Number	Sample Numbers	SssI 232 bp fragment	Tru1I 232 bp fragment	SssI 397 bp fragment	TasI 396 bp fragment
1	D1R, D3C	Morph A	Morph B	Morph B*	Morph B*
2	D1C, D3R	Morph A	Morph B	Morph A	Morph A
3	C1R, C3C	Morph A	Morph B	Morph A	Morph A
4	C1C, C3R	Morph A	Morph B	Morph A	Morph A
5	D2R, D4C	Morph A	Morph B	Morph A	Morph A
6	D2C, D4R	Morph A	Morph B	Morph A	Morph A
7	C2R, C4C	Morph A	Morph A*	Morph A	Morph A
8	C2C, C4R	Morph A	Morph B	Morph A	Morph A

Table 5.2 RFLP patterns obtained from horses used in this study.

*digestions which were different for the 2 horses with RFLP patterns distinct from the other 6.

Nine (5.1%) blood meals from a total of 178 tested were identified as not having come from the horse in the trapping net (Table 5.3). All 9 of these were trapped in the nets of repellent treated horses, not controls, although 60 samples from control nets were tested.

Sample ID	Number of mosquito blood meals not from horse in net	Could be from control horse?
D1R	2 (ABAA)	Yes
C1R	1 (AAAA)	No
D2R	1 (AAAA)	No
C2R	5 (ABAA)	Yes

Table 5.3 Mosquito blood meals not originating from the expected horse.

Consequently, as no control blood meals tested originated from a horse different from the individual in the cage, results were re-analysed considering the RFLP results (Table 5.4, Figure 5.3).

Sample	Treatment	Total number of mosquitoes	Number of blood-fed mosquitoes	Number of mosquitoes having fed on the experimental horse	Number of mosquitoes not having fed on experimental horse
D1R	DEET	6	6	4	2
D1C	Control	26	16	16	10
D2R	DEET	14	2	1	13
D2C	Control	23	12	12	11
D3R	DEET	19	0	0	19
D3C	Control	19	2	2	17
D4R	DEET	4	0	0	4
D4C	Control	46	24	24	22
C1R	Citronella	45	41	40	5
CIC	Control	36	24	24	12
C2R	Citronella	26	18	13	13
C2C	Control	59	42	42	17
C3R	Citronella	19	8	8	11
C3C	Control	55	35	35	20
C4R	Citronella	46	43	43	3
C4C	Control	72	48	48	24

Table 5.4 Total number of mosquitoes and number having taken a blood meal from the experimental horse, or not, in each sample.

Numbers underlined are those numbers which have changed from Table 5.1, due to results of blood meal analysis.

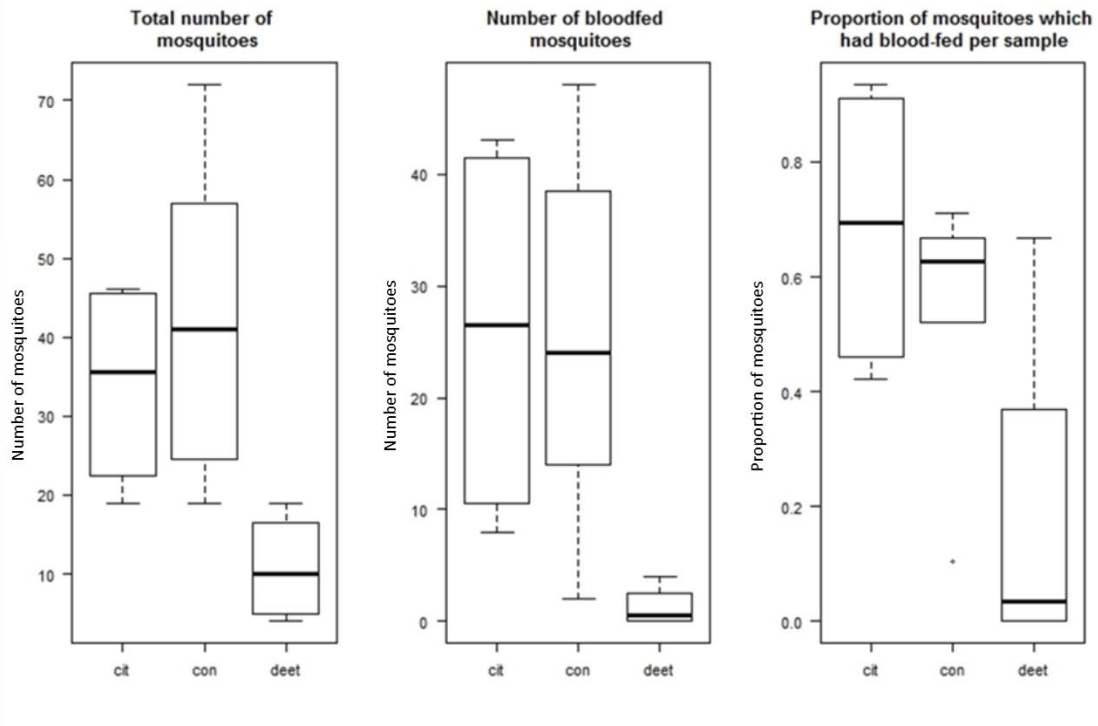

Figure 5.3 Boxplots of numbers of mosquitoes sampled and proportions of blood-fed mosquitoes confirmed to have fed on the experimental horse.

cit – Naf Off® Citronella Spray, con – control condition, deet – Naf Off® DEET Power Spray.

REVISED PERCENTAGE REPELLENCY

For DEET experiments a total of 5 mosquitoes were trapped in treatment nets, and 54 mosquitoes were trapped in control nets, giving an adjusted percentage repellency of 90.7% (95%CI; 80.1 – 96.0%) for NAF Off® DEET Power spray.

For Citronella experiments, a total of 104 blood-fed mosquitoes were trapped in treatment nets, and 149 mosquitoes were trapped in control nets, giving an adjusted percentage repellency of 30.2% (95%CI; 23.4 – 38.0%) for NAF Off® Citronella spray.

MODELLING NUMBERS OF BLOOD FED MOSQUITOES

The final model selection produced a quasi-Poisson model with treatment as a single explanatory variable. The coefficient plot and residuals plots are shown in Figure 5.4. There is a slight pattern in the residuals, and tendency towards increasing variance (heteroscedasticity), shown by fanning in the pattern of residuals, and deviation from linearity in the quantile-quantile plot. The coefficient plot shows that the standard deviation for the DEET is large compared to control or citronella. This is likely to be due to the small numbers of blood-fed mosquitoes in the DEET treatment group.

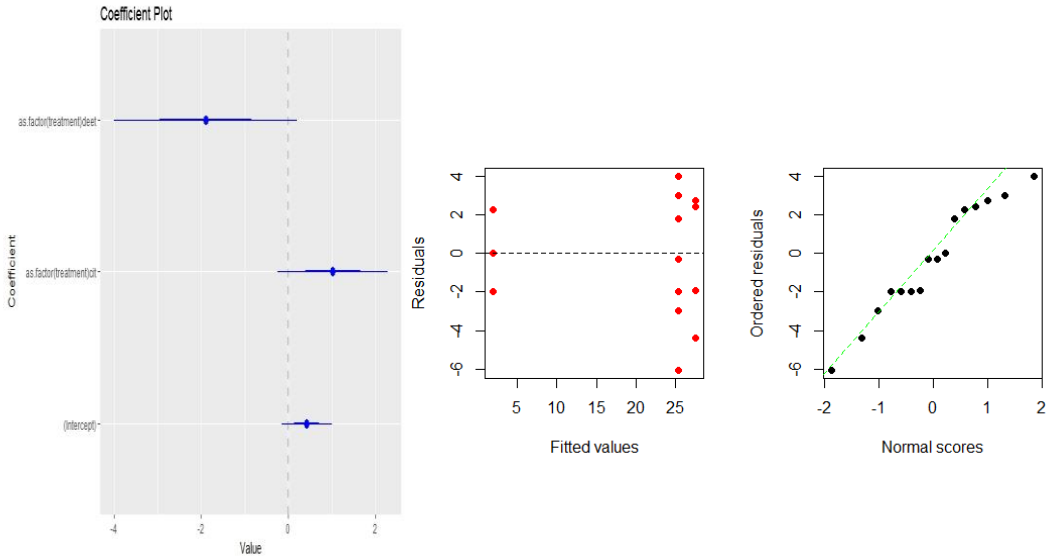

Figure 5.4 Coefficient plot and residuals plots for the final model for blood-fed mosquito numbers.

Predicted data from this model are shown in Table 5.5.

Treatment	Predicted Number of Blood fed Mosquitoes	95% Confidence Interval
DEET	2.0	0.3 - 15.2
Citronella	27.5	15.9 - 47.5
Control	25.4	17.0 - 37.9

Table 5.5 Predicted number of blood fed mosquitoes per net per night.

P-values for the comparison of treatment coefficients for the final model were <0.05 for DEET and 0.947 for citronella.

PREDICTED REPELLENCY

Calculating repellency using the predicted data (Table 5.5) from the quasi-Poisson GLM gives 95.3% (95%CI; 75.0 - 97.8) repellency for DEET. Citronella repellency was not calculated as the coefficient was not significant in the model.

MODELLING TOTAL NUMBERS OF MOSQUITOES SAMPLED

To investigate the possibility that the repellent may affect the number of mosquitoes entering the net this was data was also subjected to GLM. The final model selection produced a quasi-Poisson model with treatment as a single explanatory variable, which was used to calculate predicted values for each treatment and confidence intervals (Table 5.6). The coefficient plot and residuals plots are shown in Figure 5.5. There is a slight pattern in the residuals, suggesting a tendency towards uniform rather than normally distributed errors, shown by the slight S-shaped deviation from linearity in the quantile-quantile plot. Coefficient ranges are smaller and more uniform, due to the higher sample size compared to blood-fed counts.

Treatment	Predicted Number of Mosquitoes	95% Confidence Interval
DEET	10.8	4.9 - 23.7
Citronella	34.0	21.8 - 53.0
Control	42.0	31.7 - 55.7

Table 5.6 Predicted total number of mosquitoes per net per night.

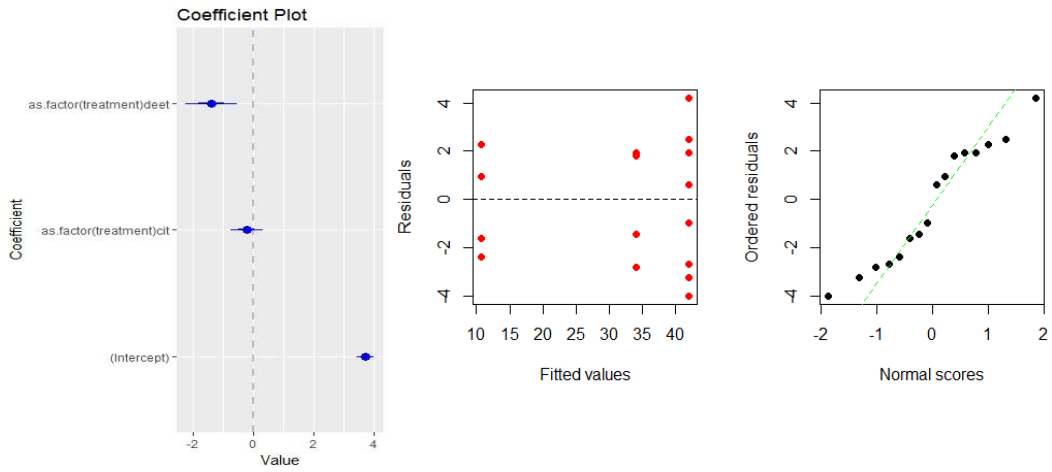

Figure 5.5 Coefficient plot and residuals plots for the final model for total mosquito numbers.

P-values for the comparison of treatment coefficients for the final model were <0.01 for DEET and 0.45 for citronella.

MODELLING PROPORTIONS OF BLOOD FED MOSQUITOES VS NON-BLOOD FED

The best fit model was quasi-binomial with treatment as the single explanatory variable. The coefficient plot and residual plots are shown in Figure 5.6. Coefficient ranges are high, especially for DEET, which is not unexpected given the small counts of blood-fed mosquitoes. A tendency towards uniformity rather than a normal distribution of errors is indicated by the quantile-quantile plot.

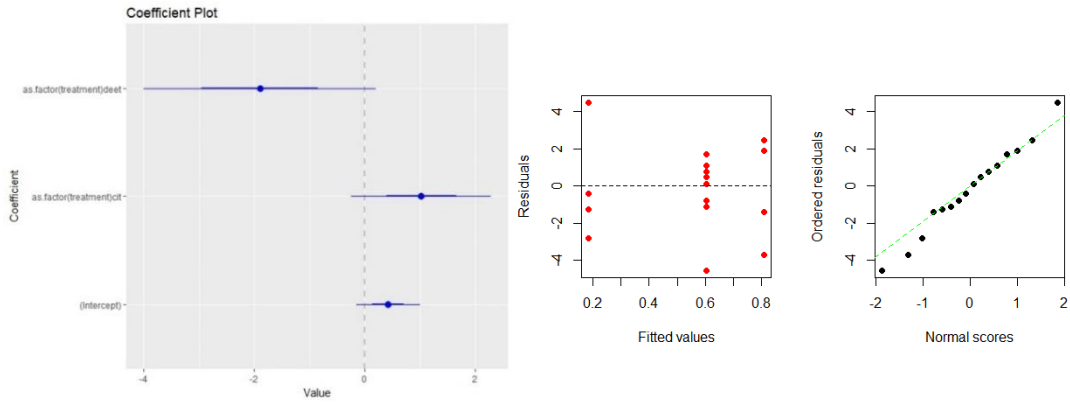

Figure 5.6 Coefficient plot and residuals plots for the final model for proportions blood fed, including blood meal analysis data.

P-values for the coefficients of individual treatments for this model were 0.0946 for DEET and 0.1313 for citronella. So, although DEET was shown to have significant effects on the number of mosquitoes entering the net and the number of blood-fed mosquitoes, it did not have a significant effect on feeding success once mosquitoes had entered the net, however, this is likely due to insufficient power of the test, hence the large standard error. This model was used to predict the odds of a mosquito being blood-fed for each treatment (DEET, Citronella, Control) and the 95% confidence intervals for these odds (Table 5.7).

Treatment	Predicted % Blood-fed	95% Confidence Interval
DEET	18.6	3.0 - 62.4
Citronella	80.8	58.4 – 92.3
Control	60.4	46.4 – 72.9

Table 5.7 Percentage blood-feds and confidence intervals for each treatment, predicted by the model.

Discussion

In the present study, neither NAF Off ® DEET Power Spray nor NAF Off ® Citronella Spray was 100% effective in preventing mosquitoes from taking a blood meal from horses used in this field experiment. However, the results for the NAF Off ® DEET Power Spray (95.3% predicted repellency) demonstrated that this product can significantly reduce nuisance biting of horses by mosquitoes. This level of repellency may provide some protection against vector-borne infections in the case of low infection pressure i.e. low vector density or very low infection rates of vectors but is unlikely to be protective in situations with high infection pressure. This study provides no evidence that NAF Off ® Citronella Spray provides any protection. In fact, the predicted number of blood fed mosquitoes and odds of a mosquito feeding successfully once it enters the net were higher for NAF Off ® Citronella Spray than for the control, but the P-value of the comparison between citronella and controls was not significant. The results of this study also confirm that UK populations of *Cs. annulata* and *An. claviger* will take blood meals from horses.

The poor performance of citronella as a repellent was expected: it has poor repellent effect in humans (Maia and Moore, 2011) and under EU Biocidal Product Regulation 528/2012, it cannot be legally marketed as a repellent for livestock, and therefore it is not marketed as such in the UK. However, this is a product commonly used by UK horse owners as an insect repellent (Chapter 3) and this is an important area of education, particularly in the event of heightened risk of equine arboviral disease.

The results for percentage repellency for DEET are higher than might have been predicted from studies in humans. In a review of efficacy trials, the mean time of protection given by 20-30% DEET for *Aedes* species is around 4-5 hours for people, and for *Ochlerotatus* spp. protection times were no more than 5 hours (Lupi et al., 2013). This may indicate that horse hair retains the repellent better than human skin. Ambient temperatures when the present study was conducted were below 20 °C and therefore sweating was probably not a significant cause of repellent loss. DEET concentrations above 15% have been shown to cause dermatosis and inflammation of the skin (in one horse after 31 days) with daily applications (Palmer, 1969) and the occurrence of hypersteatosis was correlated with the concentration of DEET applied. Repellents are not subject to veterinary medicines legislation, so before DEET were recommended as part of a protection programme against vector-borne disease it would be advisable to investigate the effects of the daily application of a formulated product to confirm a lack of significant side-effects. NAF Off ® DEET Power Spray is currently available for approximately £60 for 5 litres, so the application of

250ml (used here for larger horses) would cost £3 per application. For horses with quiet temperaments application takes less than 5 minutes for the method used. Therefore, for pet horses and horses of high value this method of protection is not necessarily prohibitively expensive or time consuming, if daily application is used for the late summer peak mosquito season. However, considering that many horses in the UK are turned out for 23 hours per day and given the timescales of protection derived from human studies, as well as the results of this study, it seems likely that re-application may be required at least twice daily, for maximum protection, which may increase the likelihood of dermatoses. For horses which are less intensively managed, of lower value, or with less amenable temperaments this method of protection is less likely to be considered practical by the owner. Quantification of DEET in hair (Martín et al., 2015) could be used to estimate appropriate time-points for field validation of protection time at 95% efficacy, to confirm how frequently application is required. Methods of application may also be important to ensure adequate coverage and retention of the repellent as in some species DEET is absorbed rapidly (Taylor et al., 1994). For example, spraying with a repellent product may not be comparable to application with a sponge (used in this study). Spray coverage may be uneven, leading to run-off or absorption and complete coverage by spray cannot be guaranteed without manual spreading of the repellent, in hard to reach places, such as the groin.

To protect individual horses from mosquito-borne disease, a high level of efficacy is required from a repellent due to the potential numbers of bites that might be sustained during a transmission period (i.e. the period for which there is transmission to horses from a wildlife reservoir, usually late in the mosquito season). As a maximum of 198 blood-fed mosquitoes were trapped in a 4-hour period during pilot trapping, it might be expected that with NAF Off® DEET Power Spray that around 9 ((100-95.3 % repellency) x 198 = 9.306) mosquitoes may have obtained blood meals from that horse in one 4-hour period. To give examples of infection rates of mosquitoes, for *Culex* mosquitoes at peak transmission of WNV in 2002 in Chicago, rates were as high as 6% of mosquitoes, so 1 in every 16.7 was infected with WNV. During JEV surveillance in Northern Australia in areas associated with pigs, mosquito infection rates were shown to be around 3% (Gu et al., 2004; van den Hurk et al., 2008). The number of expected infectious bites over a time-period of repellent use (Table 5.8) can be estimated using the average number of host-seeking females (vectors) an untreated host is exposed to, % infection rates in vectors and % repellency:

$$\frac{(1-R)\ x\ P_i\ x\ V}{T} = B_i$$

Where:

R = proportional repellency

P_i = the estimated proportion of infected vectors

V = the average number of host-seeking females the host is exposed to (i.e. the number of blood-fed mosquitoes in conditions with no repellent)

T = time period of exposure

B_i = the number of infectious bites per unit time

Equine hosts can be subjected to large numbers of mosquito bites per day as illustrated by two studies of mosquito host preference. In one study performed in the Camargue region of France, an average of 5429 mosquitoes per day obtained blood meals from a single equine host over twenty-two, 24 hour trapping periods in one season, and on average 5398 of these were known vectors of equine arboviruses (Balenghien et al., 2006). In another study an average of 148.8 blood fed mosquitoes per day were trapped using the same method in Switzerland, 38.8 of which, on average, were vectors (Schönenberger et al., 2016).

% Repellency	% of vectors infected	Number of host-seeking vectors feeding each day on untreated horses (V)	Number of infectious bites per day with repellent	Average number of days required for 1 infectious bite
95.3	1	5398[a]	2.5	0.40
95.3	3	5398	7.5	0.13
95.3	1	198[b]	0.093	10.75
95.3	3	198	0.279	3.58
95.3	6	198	0.558	1.79
95.3	1	26.2[c]	0.012	81.21
95.3	3	26.2	0.369	27.07
95.3	6	26.2	0.074	13.53

Table 5.8 Illustration of the effects of repellency on the risk per day of exposure to an infectious bite.

a – Average number of mosquito vectors obtaining blood meals each day in the study of (Balenghien et al., 2006). b – maximum number of mosquitoes obtaining blood meals in one trapping period in this study. c - average number of mosquitoes obtaining blood meals in one trapping period in this study (untreated horses).

Therefore, in the event of high levels of virus circulation and transmission in the ecosystem, equivalent to that recorded in the Carmargue, France, host-vector interaction would not be sufficiently reduced by the repellents tested, to protect against arbovirus transmission; although a satisfactory level of protection would be afforded in the UK, if mosquito densities remain as now and infection rates are not high. It is very difficult to predict at what level mosquito infection rates might occur at a particular stage of an emerging arboviral disease oubreak due to the complexity of transmission cycles of equine arboviral diseases. Concerns about increased risk of vector-borne disease outbreaks are based partially upon the expected increase in mosquito populations (vector density), but again it would be difficult to predict the increase in vector density, and therefore V, above. Given these uncertainties, and as

vaccines are available for the major diseases which cause equine fatalities, it would seem appropriate to ensure that vaccines are licensed for use in the UK in the event of increased threat to northern Europe – as is the case with the WNV vaccine.

As no peer-reviewed studies of repellent efficacy against mosquitoes for horses are available, peer-reviewed methods for assessing insecticide efficacy topically applied to livestock (Habtewold et al., 2004; Robin et al., 2015; Torr et al., 2008), and commercially used protocols for testing repellent efficacy in horses (Donahue, 2009) were considered. A research herd of horses was not available in a location with a sufficiently high mosquito density, however, a herd of horses used for commercial purposes in a suitable location was available. It was therefore imperative that the maximum number of mosquitoes could be sampled within a few hours to maximise the data obtained within a short season of high mosquito density. Observation methods used for testing fly repellency were rejected due to peak biting time of *Oc. detritus* being at dusk, and the need to differentiate mosquitoes from other flying insects. Electrified grid techniques were rejected due to practicality for use with horses, and reduction in sampling numbers due to incomplete coverage of the perimeter of the cage. Horse baited traps have been used successfully for trapping mosquitoes and other vectors in a number of studies of host-vector interaction (Balenghien et al., 2006; Schönenberger et al., 2016; Viennet et al., 2011), and therefore these methods were applied in the present study. This method of testing individual protection methods for horses has been shown to be practical for use in locations with moderate to high vector density.

Testing of insect repellency using rugs and masks impregnated with permethrin was considered. However, it seemed unlikely given the incomplete coverage of horses that is provided by these rugs, that adequate efficacy levels could be achieved to be protective from infection as previously discussed. In addition it has been shown that topical pyrethroid insecticides, in general, are unable to prevent infected bites to the individual due to the time it takes for knock-down to occur although they may reduce biting rates (by excito-repellency (Hossain and Curtis, 1989)) in one study in cattle, by approximately 50% (Habtewold et al., 2004). Vector mortality after feeding on horses, and its potential effect on reducing ongoing transmission, is only relevant for epidemic VEEV, as the horse is a dead-end host for the other arboviruses discussed in this thesis.

The main limitations of the present study are caused by the limited number of trapping nights within the late, short period of peak mosquito density, and weather conditions during that peak in 2016. Sampling was curtailed in mid-October due to persistent wind and rain. Experiments were additionally challenging due to the need for horses to remain dry and without rugs on during the 7-hour period between repellent application and testing, and the need to work with the correct pair of horses on each particular day. Therefore, it was not

possible to test more than two treatments. Blood meal analysis using RFLP as described here was not able to discriminate between all horses used in the study. Sequencing of PCR product from the two hypervariable regions amplified could be tested as a cost-effective alternative. The other methods, single nucleotide polymorphism analysis (Hirota et al., 2010) and microsatellite marker analysis (Binns et al., 1995), which is available commercially in the UK (Weatherbys Scientific, 2018), are suitable but incur greater costs.

The results of the present study show that 20% DEET can significantly reduce nuisance biting by mosquitoes and may be of some benefit in reducing the risk of vector-borne disease to individual horses where there is low infection pressure. However further work to estimate protection time is required. NAF Off® Citronella Spray was ineffective in significantly reducing biting. Other repellents such as icaridin, which is also available for use in horses, should be tested. It shows good efficacy in humans and is not known to cause dermatological side-effects in horses. However, the licensing of vaccines widely used in affected countries, for the protection of horses, should be considered the main priority in the event of increased risk of equine arbovirus transmission in the UK.

6 LABORATORY-TESTING REPELLENTS FOR THE PROTECTION OF HORSES FROM MOSQUITO BITING

Abstract

In the event of increased risk of transmission of equine arboviruses in the UK, particularly for those diseases for which no vaccines are available, individual horse protection will be of great importance to horse owners. It is therefore critical to know which protection methods are most effective against UK mosquitoes. Laboratory screening methods are required to test candidate products and new active ingredients, before field testing. A laboratory and semi-field study was conducted, to test 6 products available on the UK equine market. Wild-caught *Oc. detritus* in small testing cages were observed for landing and probing behaviours when exposed to the products tested, using human volunteers as bait, and then using horses as bait. Icaridin-based Red Zone Super Spray gave the highest percentage repellency in human trials (93.8%, 95%CI; 83.2 – 97.9%) followed by NAF Off ® DEET Power Spray (87.5%, 95%CI; 73.9 – 94.5%), then P-menthane-3.8-diol-based NAF Off ® Extra Effect Spray (86.7, 95%CI; 77.2 – 92.6%). Therefore these 3 products are potentially useful repellents for the protection of horses from biting by *Oc.detritus*. Results suggest a reasonable agreement between the present study and the field study described in Chapter 5, although more work is required to confirm this. Human bait trials described in this chapter appear to be a suitable screening method to reduce the number of horse experiments required to test repellent efficacy against mosquitoes. The horse baited trials described require further refinement to be useful as a screening method for repellent efficacy.

Introduction

In Chapter 2 it was demonstrated that potential mosquito vectors are present on equine premises in the UK, and in Chapter 5 the importance of control methods to reduce mosquito biting in the event of disease transmission was discussed. Horse-level measures for reduction in the risk of arboviral infection to the individual horse rely on either vaccination or mosquito bite prevention or reduction. Vaccinations are available worldwide for most of the major arboviruses (VEEV, EEEV, WEEV, WNV, JEV, Getah virus) but not for MVEV, RRV. If an outbreak of one of these diseases were to occur, mosquito control and protection from mosquitoes would be critical in minimising risk to the individual horse. In Chapter 3, repellents were shown to be the most common bite-reduction method used by horse owners, and in Chapter 5 both the success and challenges of field-testing mosquito repellents on horses were demonstrated.

Field testing of mosquito repellents on horses is time-consuming, has ethical implications, and is expensive. Mosquito density and weather in the UK is unpredictable compared to that in many other climates (e.g. tropical), where the majority of repellent testing is undertaken. The option of releasing mosquitoes into a large cage environment with horses as bait (Blume et al., 1973) was considered, as this would have enabled vector density to be guaranteed for each test, and allowed for the use of facilities including climate and lighting control. However, this would have required a Home Office licence and was rejected on ethical grounds. Even though repellent testing in this study is for the benefit of horses, and field testing was minimally disruptive to the horses' routine, it was still considered important to be able to minimise equine field tests for ethical reasons as well as practical ones. Ideally then, rapid laboratory-based techniques, which are less expensive than field testing, could be used to determine the potential efficacy of new repellent compounds or products, using European mosquito species. Two techniques were considered to be potentially useful tests: the use of a human bait as a model for efficacy in equines, and the use of mosquitoes in small cages which could be recorded attempting to bite, whilst being physically prevented from successfully biting the test horse.

The aim of this study was to investigate which of several spray repellent products, readily available on the UK equine market, were most likely to protect horses, based on laboratory trials, and to compare methods for doing so. The objective was to determine which products should be further investigated in field trials with horses, using the methods described in Chapter 5. The underlying hypothesis was that products which perform well in testing for protection of humans, would demonstrate higher efficacy than those which do not perform

well in studies on human protection: products based on DEET and icaridin would perform well, and those based on citronella and neem would not.

Materials and Methods

Ethical permission for equine participation in this study was obtained from The University of Liverpool Veterinary Ethics Committee (VREC444). Due to the fact that *Oc. detritus* may cause moderate to severe localised reactions following biting of test subjects, and wild-caught mosquitoes cannot be guaranteed to be pathogen-free, it was considered inappropriate to allow mosquitoes to bite the human bait. Therefore, chambers that physically separate the mosquito from the human or horse bait were used. Volunteers were University of Liverpool staff or postgraduate students who were experienced in mosquito husbandry, and who provided informed consent to participate in the study.

6.3.1 Mosquitoes

Experiments were conducted on *Oc. detritus* adults that were collected as larvae or pupae from salt marshes on the Wirral Peninsula, North West England. Immature mosquitoes were reared in ambient conditions in water collected from their larval habitat, supplemented with tap water as necessary. Where supplementary food was required Brewer's Yeast was provided. Adults were allowed to emerge and mate in 30 × 30 × 30 cm BugDorms (BugDorm, Taichung, Taiwan). They were kept in ambient conditions until testing and were offered 10% sucrose. Sucrose was removed 24 hours before testing. Thirty adult females were transferred to a testing cage at least 30 minutes before testing. Testing cages were Bugdorm-4 Rearing Cages (Model BD41515, BugDorm, Taichung, Taiwan) measuring 17.5 x 17.5 17.5 cm. These cages are made of polyester netting and have a clear plastic side panel.

6.3.2 Repellents

The repellents tested were readily available spray products on the equine market in the UK, or products commonly used for the purposes of repelling flies, by UK horse owners (Chapter 3):

- NAF Off ® DEET Power Spray (NAF, Monmouth) containing 19.6% w/v N,N-Diethyl-m-toluamide (DEET)

- NAF Off ® Citronella Spray (NAF, Monmouth) containing an undisclosed quantity of citronella oil
- NAF Off ® Extra Effect Spray (NAF, Monmouth) containing 1% w/v P-menthane-3.8-diol (PMD)
- Power Phaser (Leovet, Lahnau, Germany) containing 5.51% w/v DEET and 4.91% w/v IR3535 (Ethyl Butylacetylaminoproprionate)
- Red Zone Super Spray (Red Horse Products, Henley-on-Thames) containing 20% Saltidin (icaridin) and undisclosed amounts of bog myrtle oil, cade oil, garlic oil and lemon eucalyptus oil
- 2 in 1 Ultimate Fly Repellent and Skin tonic (Stable Environment, Halifax) containing undisclosed amounts of Neem oil and Lemon Eucalyptus oil

50% w/v DEET (Sigma Aldrich, Gillingham, UK) in ethanol, was used as a positive control.

6.3.3 Pilot Work

Pilot work involved trialling several different testing set-ups including different designs of testing cage and using repellent directly on human skin, with a 0.5 cm separation from mosquitoes. Finalising the experimental set-up to maximise measurable mosquito response to bait, and to produce high enough quality video for post-testing analysis was challenging. Various video equipment including a GoPro HERO 3 and various macro lenses were trialled. However, the depth of field (distance between the nearest and furthest object giving a focused image) and the amount of detail required to analyse the probing behaviour of mosquitoes across an adequate testing area, required high-quality equipment. Therefore, a DLSR camera with a macro lens and tripod was obtained (Nikon D7000 DSLR camera with a Nikon 55mm Micro Nikor fixed focal macro lens). All preparations and experiments involving horse hair took place in outdoor facilities due to restrictions regarding animal allergens precluding its use in laboratories. Additional lighting was required to maintain an adequate depth of field. Four lamps were used to increase and to help to standardise light levels. They were placed to provide a diffuse light around the testing cage and the response of mosquitoes was compared to the response of mosquitoes to human bait in good natural lighting in a laboratory. No difference in the response of *Oc. detritus* in these two conditions was noted. Video recording of horse testing proved impractical due to the quality of camera equipment required.

6.3.4 Human bait experiments

EXPERIMENTAL DESIGN

The aim of this study was to screen formulated products for efficacy, before further testing in a field setting (Chapter 5). Where formulated products are tested for complete protection time this requires 200-250 mosquitoes every 30-60 minutes for each volunteer. Therefore, repellent efficacy testing was based only on percentage efficacy in this study, due to the large number of mosquitoes required otherwise, and the need to test several products. For each product, it was planned to undertake 4 replicates with each volunteer or horse.

EXPERIMENTAL SET-UP

Two adult volunteers (1 male and 1 female) were used to test products after 6 hours. One ml of product was added to 1 g of hair horse hair in a petri dish. Horse hair was all obtained from the clipping of one horse, during early winter coat growth. The hair had been stored in a zip-lock bag for 2 months before use, in the outdoor facilities where testing took place. Hair and repellent were mixed using a gloved finger, then left to dry for 6 hours on a clean polyester net. After the appropriate drying time, the hair was placed onto a new net, so that only the effect of product dried onto the hair would be measured. Nets were held flat by stretching them in an up-turned Donut Lid supplied with pint-sized BugDorms (Part BCC0001, BugDorm, Taichung, Taiwan).

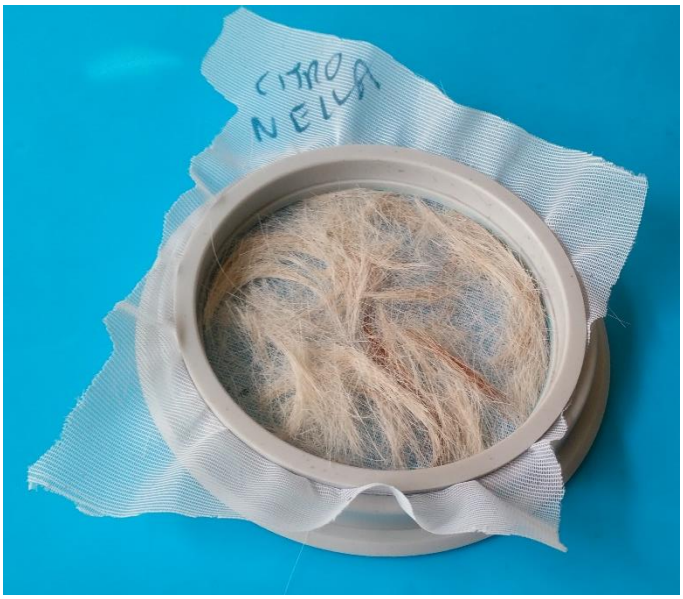

Figure 6.1 Donut lid with netting and horse-hair.

Testing cages were prepared by using the Donut Lid to create a marker outline for the testing area, which was visible from the underside of the net (Figure 6.2).

Figure 6.2 Snapshot taken from video, showing mosquitoes probing in testing area (blue circle).

Mosquitoes were placed in testing cages and were moved to the testing facility at least 30 minutes before testing to acclimatise. Before each product test, each cage of 30 mosquitoes was tested for response to the bait. This control response was measured using untreated horse hair (1 g) on the net. The Donut Lid holding the net was placed onto the mesh lid of the testing cages (described previously) and held in place with 4 pins, to confirm correct placement for video analysis (Figure 6.3).

Figure 6.3 Images showing testing cages including pins, and cage marking to ensure correct testing area used

TESTING

For each control and product test, the volunteer's hand was placed on top of the Donut Lid (Figure 6.4). The area outlined by the Donut Lid and associated pen mark on the underside

of the net defined the testing area. Mosquitoes were stimulated to attempt to feed by breathing on the cage for 15 seconds immediately before the measurement period (carbon dioxide stimulus). A Nikon D7000 DSLR camera with a Nikon55mm Micro Nikor fixed focal macro lens was used to take a 60-second video with the hand in place above the horse hair for the full test period. If less than 10 mosquitoes were observed moving and showing interest in the bait (i.e. appeared to be probing), the cage was discarded and a new cage of 30 mosquitoes was used. Immediately after the control video was finished, the net with the untreated horse hair was removed and replaced with a net containing treated horse hair. The procedure was repeated with treated horse hair on the same cage of mosquitoes.

Figure 6.4 Image of testing-cage set-up.

VIDEO ANALYSIS

For each replicate of a product test, two 60 second videos were analysed: the pretest control, and the product test. For each 60 second video (Figure 6.2), 4 values were recorded: number of probing events (a mosquito beginning to probe, for the first time, in the test area), number of landing events (a mosquito landing in the test area), maximum number of mosquitoes probing in the testing area at any one time, maximum number of mosquitos resting in the testing area at any one time. The number of mosquitoes already resting on the testing area at the beginning of the test period were not counted as landing events but were counted for

probing if this behaviour was observed. The testing area for analysis was defined by the circular area marked previously on the upper cage netting. The open-source event-logging software, BORIS (Friard and Gamba, 2016), was used to record probing and landing events. Blinding of video identity was required to prevent the order of alternate control and treatment videos from affecting the analysis. Blinding was achieved by renumbering videos, using random sampling of a numerical list produced by the sample function in R (R Core Team, 2017). Videos were then analysed in this random order. Repeatability was checked by repeating counts on 10 videos the day after they were originally analysed. Videos with moderate to high mosquito response were chosen as these were considered likely to have the lowest repeatability due to higher counts. Counts of all measures were identical for all re-analysed videos. Results were de-coded once all videos had been analysed.

6.3.5 Horse bait experiments

ANIMALS

The selection criteria used for recruitment were that the horses were healthy (with no history of a skin condition) and did not have a history of being treated with any topical insecticide within the last two months and no repellent or worming treatment within the previous month. Horses were owned by the same individual and ranged in age from 6 years to 15 years. All horses were habituated to the testing cages before testing took place, by placing empty cages against their flank.

EXPERIMENTAL SET-UP

The experimental design of the horse testing was similar to that of the human bait tests described previously. The same 3 horses were used to test each product. The time point for repellency testing in this study was 4.5 hours. This was necessary due to the availability of mosquitoes coinciding with the field-repellency study undertaken in Chapter 5. It was not possible to use a longer time point and undertake the field study at the same time.

Testing cages were modified by attaching the Donut Lid (both described previously) to the centre of one of the sides of the cage. This addition, as well as the design of the cages, ensured that although mosquitoes could be observed probing to try and bite a horse, they were never able to contact the horses' skin.

REPELLENT APPLICATION

Horses were prepared for repellency testing by clipping two small perpendicular marks on one flank. The repellent to be tested was applied 4.5 hours before the test was due to take place. A small sponge was used to apply repellent to a patch 20 cm square below and right of the clip marks (so that the clip marks formed the corner of the square patch). Initially 2 ml was used, and up to 5 ml if this was not enough to dampen the 20 cm square patch of hair. The positive control 50% DEET was not used in horse testing due to the possibility of this causing dermatological side-effects.

TESTING

Two researchers, experienced in horse handling, carried out testing with horses. Horses were not tied up but were restrained with a head collar and lead-rope. Testing was abandoned if the horse became distressed at any time, and a replacement horse was used. Testing took place outside to ensure adequate lighting. A sheltered location was used to minimise any effects of the wind on mosquito responses. For the control pretest, the mosquito cage was placed against the untreated flank of the horse and the number of probing events and landing events in the testing area was recorded for a 30 second testing period. The same cage of mosquitoes was then placed on the repellent treated patch on the opposite flank of the horse with the corner of the cage aligned with the clip marks. The number of probing and landing events were recorded for 30 seconds. Immediately before each 30 second recording period (both control pre-test and treatment), carbon dioxide stimulus was provided to the mosquitoes by means of the researcher breathing on the cage for 15 seconds.

6.3.6 Data Analysis

Percentage repellency and efficacy for prevention of landing events, and reduction in the maximum number of mosquitoes probing and resting were all was calculated using Abbott's formula (Abbott, 1925):

$$\%\, repellency = 100\, x \frac{(C - T)}{C}$$

Where C = pre-treatment control count

T = treatment count

Confidence intervals for a proportion were calculated using the Wilson method (Sergeant, 2017b). The difference in two proportions was analysed using Pairwise Fisher's Exact Test with a holm correction (Holm, 1979) (pairwise.fisher.test – fmsb package (Nakazawa, 2017)) in the R statiscal programming environment (R Core Team, 2017).

Results

6.4.1 Human bait experiments

Once videos were analysed it was apparent that mosquito control responses were not as aggressive as initially thought. Of 56 tests, only 24 fulfilled the criteria that control responses should include at least 10 probing events (Table 6.1). The minimum number of replicates, in the final analysis, was 2 for 50% DEET and NAF Off ® DEET Power Spray.

	Treatment				Control			
Treatment	**Probing events**	**Landing events**	**Maximum No. Probing**	**Maximum No. Resting**	**Probing events**	**Landing events**	**Maximum No. Probing**	**Maximum No. Resting**
50% DEET	2	1	1	2	23	5	19	19
	3	3	2	4	38	21	11	13
NAF Off ® Citronella Spray	0	0	0	0	10	2	8	8
	15	9	8	8	26	15	8	10
	2	2	1	1	14	6	9	9
NAF Off ® DEET Power Spray	4	5	2	2	27	17	11	11
	1	1	1	1	13	6	8	9
NAF Off ® Extra Effect Spray (PMD)	2	2	1	1	12	8	6	7
	0	0	0	0	11	2	7	8
	4	3	2	2	10	5	5	6
	1	0	0	0	22	9	10	10
	1	0	2	2	10	7	4	6
	2	1	1	2	10	5	4	4
2 in 1 Ultimate Fly Repellent and Skin tonic (Neem)	4	4	2	3	16	7	13	15
	3	3	1	1	12	4	10	12
	1	1	1	2	15	7	10	10
Power Phaser (DEET + IR3535)	7	1	5	5	10	6	5	5
	15	4	13	15	19	2	14	16
	5	6	3	3	35	15	14	15
	3	3	2	3	17	8	8	9
Red Zone Super Spray (Icaridin)	0	0	0	0	15	3	10	10
	0	3	0	1	19	9	11	11
	3	3	1	2	14	6	8	9

Table 6.1 Results for human bait experiments, with control responses greater than 10 probing events

These results were used to calculate percentage repellency using probing events, presented in Table 6.2.

Product	Percentage Repellency (95%CI)
50% DEET[a,b]	91.8 (82.2 – 96.4)
NAF Off ® Extra Effect Spray (PMD)[c]	86.7 (77.2 – 92.6)
NAF Off ® Citronella Spray[a,d]	66.0 (52.2 – 77.6)
Red Zone Super Spray (Icaridin)[d,e]	93.8 (83.2 – 97.9)
NAF Off ® DEET Power Spray	87.5 (73.9 – 94.5)
2 in 1 Ultimate Fly Repellent and Skin tonic (Neem)	81.4 (67.4 – 90.3)
Power Phaser (DEET + IR3535)[b,c,e]	63.0 (52.1 – 72.7)

Table 6.2 Percentage repellency calculated from probing events in human bait experiments.

Superscript letters denote treatments between which there is a significant difference in efficacy. a – P-value <0.05, b – P-value <0.01, c – P-value <0.05, d – P-value <0.05, e – P-value <0.01

In this study, the positive control 50% DEET did not give 100% repellency after 6 hours. Icaridin-based Red Zone Super Spray had a percentage repellency of 93.8% (95%CI; 83.2 – 97.9%) which was slightly higher than that of 50% DEET, but confidence intervals for all products were wide, ranging from around 15 – 25%. Power Phaser gave the lowest percentage repellency (63.0%, 95%CI; 52.1 – 72.7%), similar to that of NAF Off ® Citronella Spray (66.0%, 95%CI; 52.2 – 77.6%). Due to the small number of successful trials for some treatments, including the positive control, it was not considered appropriate to attempt to use generalised linear modelling to analyse these data.

To investigate whether the other measured parameters (Landing Events, Maximum Number Probing and Maximum Number Resting) produced similar results and to illustrate the variation in results from each trial, a scatterplot of the efficacy of the repellent in each trial for each parameter was produced (Figure 6.5). From these plots it appears that the performance of Power Phaser and NAF Off ® Citronella Spray was less consistent in the trials than other products, corresponding to a wider range 95% CI, for repellency. The performance of PMD-based NAF Off ® Extra Effect Spray is also somewhat inconsistent across all outcomes.

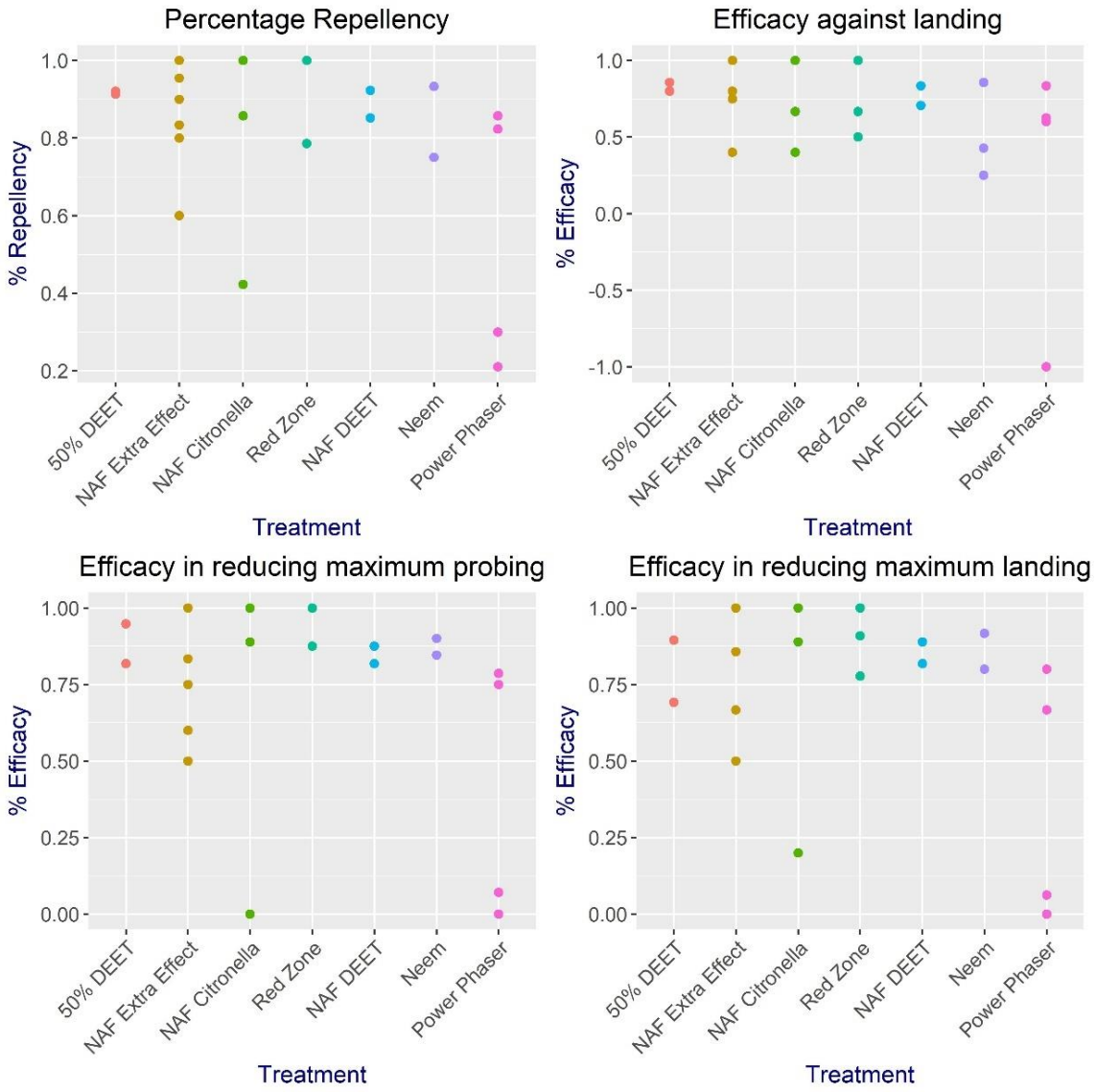

Figure 6.5 Scatterplot showing efficacy results for each trial and treatment.

Neem - 2 in 1 Ultimate Fly Repellent and Skin tonic.

When efficacy in preventing landing events was compared, there were no significant differences between treatments. Visual assessment of all the outcome parameters indicates that they are in broad agreement. Therefore, as the aim of this experiment was to determine repellents which should be investigated in more detail with field tests: Red Zone Super Spray, NAF Off ® DEET Power Spray and NAF Off ® Extra Effect Spray appeared most likely to provide some protection and were candidates for further investigation.

6.4.2 Horse bait experiments

The results of horse bait trials are presented in Table 6.3. However, during testing, it became apparent that there was no clear effect, even for products expected to give a high percentage repellency (Red Zone Super Spray and NAF Off ® DEET Power Spray). Testing was

suspended after 1 trial for each horse, as results were of poor quality and therefore continued horse-baited testing was considered inappropriate.

Treatment	Control		Treatment	
	Probing Events	Landing Events	Probing events	Landing Events
NAF Off ® DEET Power Spray	13	34	8	13
	18	20	4	11
	12	13	6	11
Power Phaser (DEET + IR3535)	7	14	2	5
	8	10	6	10
	8	10	7	8
NAF Off ® Extra Effect Spray (PMD)	10	12	12	15
	3	4	3	3
	7	8	7	8
2 in 1 Ultimate Fly Repellent and Skin tonic (Neem)	18	10	4	6
	7	14	6	12
	6	13	3	5
Red Zone Super Spray (Icaridin)	11	4	10	3
	17	7	2	12
	14	5	6	7
NAF Off ® Citronella Spray	14	5	5	3
	20	5	8	4
	9	5	2	3

Table 6.3 Results of horse-baited repellency trials.

Percentage repellency was below 70% for all products (Table 6.4) and confidence intervals ranged between 27 and 37%. Interpretation of these results is slightly complicated by the fact that the positive control 50% DEET could not be used on horse skin. However, as NAF Off ® DEET Power Spray contains 19.6% DEET, and was also tested on horses in the field in Chapter 5, giving a percentage repellency of 95.3%, it could be considered an acceptable substitute.

Product	Percentage Repellency (95%CI)
NAF Off ® Extra Effect Spray (PMD)	No repellent effect
NAF Off ® Citronella Spray	65.1 (50.2 – 77.6)
Red Zone Super Spray (Icaridin)	57.1 (42.2 – 70.9)
NAF Off ® DEET Power Spray	58.1 (43.3 – 71.6)
2 in 1 Ultimate Fly Repellent and Skin tonic (Neem)	58.1 (40.8 – 73.6)
Power Phaser (DEET + IR3535)	34.8 (18.8 – 55.1)

Table 6.4 Percentage repellency calculated from probing events in horse-baited experiments.

This positive control then, gave only a percentage repellency in horse bait experiments of 58.1% (95% CI; 42.2 – 70.9), compared to 87.5% (95% CI; 73.9 – 94.5) in human bait experiments and 95.3% (95% CI; 75.0 - 97.8) in the field study. This also indicates that the results of these horse bait experiments are unlikely to be reliable. The variation in efficacy produced by these experiments was very high as illustrated by the scatter plot Figure 6.6, and the standard deviations reported.

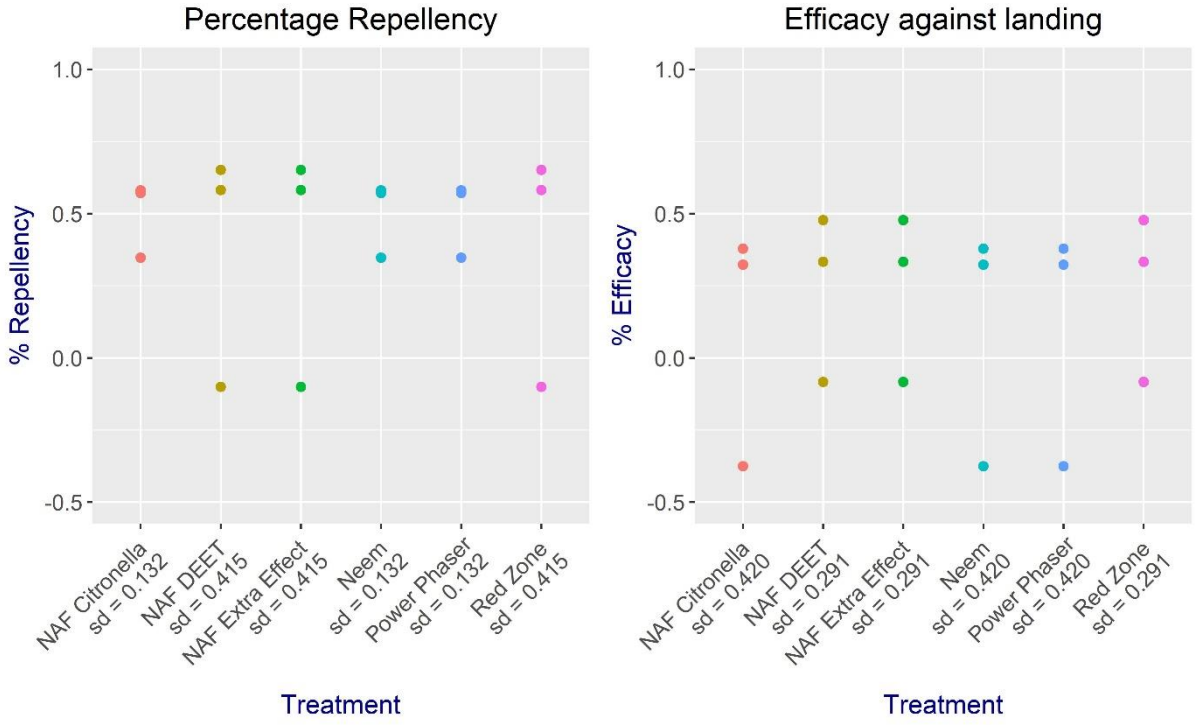

Figure 6.6 Scatterplot of efficacy results for individual horse-baited trials.

Neem – 2 in 1 Ultimate Fly Repellent and Skin tonic.

Comparing the standard deviation between the human bait experiments and horse bait experiments (Table 6.5), it is apparent that variance was generally higher in the horse bait experiments. Removing the trials where control probing response was less than 10 resulted in a standard deviation of 0.202 NAF Off ® DEET Power Spray and 0.399 for Red Zone Super Spray. This was not considered an adequate correction to provide reliable data.

Product	Standard deviation in Human Bait experiments	Standard Deviation in Horse Bait experiments
NAF Off ® Extra Effect Spray (PMD)	0.142	0.415
NAF Off ® Citronella Spray	0.300	0.132
Red Zone Super Spray (Icaridin)	**0.124**	**0.415**
NAF Off ® DEET Power Spray	**0.050**	**0.415**
2 in 1 Ultimate Fly Repellent and Skin tonic (Neem)	0.106	0.132
Power Phaser (DEET + IR3535)	0.340	0.132

Table 6.5 Standard deviation in calculated repellency for human-baited and horse-baited trials.

Results of horse bait experiments were not analysed further as the data were considered unreliable.

Discussion

In the present study none of the tested products, nor the positive control (50% DEET in ethanol) gave 100% repellency. In human-baited trials there was a significant difference in repellency between the positive control and both NAF Off ® Citronella Spray and Power Phaser, indicating that these are significantly poorer repellents than 50% DEET. Icaridin-based Red Zone Super Spray gave the highest percentage repellency in human trials (93.8%, 95%CI; 83.2 – 97.9), followed by NAF Off ® DEET Power Spray (87.5%, 95%CI; 73.9 – 94.5), then PMD-based NAF Off ® Extra Effect Spray (86.7, 95%CI; 77.2 – 92.6). Horse baited trials did not appear to produce reliable data in this study.

The results of human-baited trials for this study for icaridin-based Red Zone Super Spray, NAF Off ® DEET Power Spray and PMD-based NAF Off ® Extra Effect Spray were in broad agreement with those of human trials in previous studies, in which icaridin performance is comparable with that of DEET, depending on the mosquito species. PMD has also been shown to be an effective repellent for human use in many circumstances (reviewed in Lupi et al., (2013)).

As the aim of this study was to enable recommendation of products to test in field trials, a comparison between the results of the present study and field trials is key. In Chapter 5, two products were tested: NAF Off ® DEET Power Spray with a percentage repellency predicted using generalised linear modelling of 95.3% (95%CI; 75.0 - 97.8), or 90.7% (95%CI; 80.1 – 96.0%), calculated from raw data, and NAF Off ® Citronella Spray with a percentage

repellency of 30.2% (95%CI; 23.4 – 38.0%). The results for NAF Off ® DEET Power Spray obtained in the present study in human trials (87.5%, 95% CI; 73.9 – 94.5), compare well with the results of field trials. For NAF Off ® Citronella Spray with 66.0% (95% CI; 52.2 – 77.6) in the present study, the results are less similar. It is not clear why NAF Off ® Citronella Spray should appear to have a stronger repellent effect in this laboratory study than in the field study described in Chapter 5. However, host location and feeding stimulation of mosquitoes involved a number of cues, including carbon dioxide plumes, volatiles such as 1-octen-3-ol, and visual stimuli (Gibson and Torr, 1999; Torr et al., 2008). These stimuli are likely to be more pronounced factors in field testing with livestock, than in this laboratory design, with a human host.

Testing of repellents on the host intended to be protected (usually humans) is generally the method of choice. *Ochlerotatus detritus* are aggressive biters of a wide range of mammals including humans (Service, 1971b) and horses (Chapter 5), and the proximity of a human host in the insectary generally produced an obvious probing response in this wild-caught mosquito. Therefore, it was considered that the problems associated with testing repellent efficacy against anthropophagic mosquitoes using laboratory animals were unlikely to be relevant in human baited trials conducted during this study. In addition, factors such as body temperature, hair length and density and skin permeability (Rutledge et al., 2015) were unlikely to be relevant as neither the mosquito nor the repellent was in contact with human skin. However, volatiles produced on the breath and from skin, odour, size and volume of carbon dioxide produced, are obvious differences between these two hosts and it is not known how these might affect the efficacy of repellents between hosts, either in the laboratory or in the field. In addition, because horses' hair coat length and density varies between horses, and between anatomical regions, it is not known how this may affect repellent efficacy. To the author's knowledge, there have been no studies comparing the effect of repellents between different species of natural hosts, for mosquito species with a broad host range.

Whilst a repellent may reduce biting without reducing landing or probing (Rutledge et al., 2015), either landing or probing may be used according to the WHO guidelines for repellent testing (WHO, 2009), and the recording of both may be important for the interpretation of the test, where a repellent prevents probing but not landing. In the present study, there were no repellents for which it was obvious that this was the case. In using video analysis of mosquito behaviour, it was apparent that at least for the more effective repellent products, there were often a number of mosquitoes that landed and began to probe but then quickly desisted, suggesting that close contact of the proboscis with the repellent treated hair was necessary for the repellent action. It was also noted that even for the most effective

repellents, not only were there still probing events but often a small number of mosquitoes would probe for long periods without apparently being affected by the repellent.

Technologies are now available to track mosquitoes (Manoukis et al., 2014), and accurate measurement of time spent resting on a surface can be measured (Parker et al., 2015), although the automatic recording of probing may still prove challenging. There have been no studies focussing specifically on the probing behaviour of mosquitoes in response to repellent treated hosts or surfaces. However, the advantages of such intimate behavioural studies of mosquito responses on our future understanding of repellent and insecticide modes of action are increasingly recognised as being important (Angarita-Jaimes et al., 2016; Baldacchino et al., 2013; Spitzen et al., 2014). Similar studies including probing behaviour, as well as flight and landing behaviour should be conducted in future, as technologies allow, in order to increase our understanding of behavioural mechanisms of repellent resistance and allow for cost-effective screening of products for individual protection.

The main limitations of this study were due to small sample sizes. It became apparent in the analysis that although control mosquito responses appeared sufficient (during testing) to provide 10 probing events per sample, once videos were analysed this was often not the case. Due to restrictions in the period of mosquito availability for the 3 main experimental studies in this thesis (Chapter 4 and Chapter 5 and this study), it was not possible to complete video analysis in synchrony with conducting the experiments. Therefore, failure to produce 8 ideal replicates for each product was not apparent at the time. In this study, 2 human volunteers were used and at least one more would have been ideal. The use of hair from different horses in a cross-over design would also have improved the experimental design. However, it was not considered that the odour of the horse would be remaining on the hair after storage. Therefore, the hair was not being used as part of the host bait, rather it was being used solely to approximate the persistence of repellent on a horse, and to provide an appropriate medium through which the mosquitoes should probe. The number of mosquitoes required to implement such a cross-over design with more human volunteers and the same number of products tested precluded these improvements, particularly as this study was undertaken concurrently with the study in Chapter 4, also using *Oc. detritus*.

Horse baited trials were not successful, though refinement of the experimental protocol may improve reliability, if this study were to be extended in future. In pilot work for human baited trials, it was noted that recordable control responses were higher when the testing area was on the top surface of the cage rather than on one of the sides. This presents difficulties when using the equine host as bait, in that the ventrum (belly) of the horse would become

the contact point. This is more likely to create safety concerns due to equine temperament than using the flank and makes it more challenging to count mosquito responses, as well as reducing the separation between control and treatment areas on the horse's body. Another potential cause of variation in mosquito responses in the horse baited trials is the wind, as experiments were undertaken outside due to the lack of well-lit indoor facilities at the field site. Future work to validate or discount this method as a useful screening technique for repellent efficacy could include repeating these experiments with a higher number of replicates, indoors, and with a ventral contact point on the host. Smaller cage designs should be investigated for use on the ventrum.

The results of the present study identified icaridin-based Red Zone Super, DEET-based NAF Off ® DEET Power Spray and PMD-based NAF Off ® Extra Effect Spray, as potentially useful repellents for the protection of horses from mosquito bites. This information is important should there be heightened risk of mosquito-borne disease in horses in the UK. Results suggest a reasonable agreement between the present study and the field study described in Chapter 5. NAF Off ® DEET Power Spray was shown to provide a similar percentage repellency in both studies. Red Zone Super Spray and NAF Off ® Extra Effect Spray should be tested in the field to confirm efficacy in reduction of mosquito biting on horses in field conditions. Human baited trials described in this study are a useful screening technique, but not a substitute for field testing of repellents for use in horses.

7 Final Discussion and Conclusions

General Discussion

The future risk to horses in the UK from mosquito-borne arboviruses is dependent upon many factors ranging from the level of movement of potentially infected animals or people worldwide and the extent of surveillance and control measures implemented in affected countries, to vector density and vector-equine interaction, and outbreak recognition and control in the UK. The focus of this thesis was to investigate the risk to UK horses, should a successful virus introduction event occur, with regards to equine-vector interaction, vector competence of mosquito species native to the UK and efficacy of protection methods.

8.1.1 Virus Introduction Risk

Assessing the risk of virus introduction in the UK, and Europe as a whole, is challenging. For example, the classical ecological niche for endemic JEV (proximity of swine and rice culture) does not exist in the UK. However much European rice production takes place in Italy and Spain (Global Rice Science Partnership, n.d.) and both rice and pig production take place in Northern Italy (European Commission, 2014; United States Department of Agriculture, 2017). This was also the area where a partial genome sequence of JEV was detected in *Cx. pipiens* in 2010 (Ravanini et al., 2012) although no further evidence of JEV has been found since. Nothing is known about potential reservoir hosts for endemic VEEV in Europe although exotic pet imports are considered a risk for virus introduction (Durand et al., 2013; Pages et al., 2009) and possibly people as well as horses are potential transport hosts for epizootic VEEV (Morrison et al., 2008). EEEV has been suggested as a virus with high introduction potential, compared to other arboviruses, in Europe (Durand et al., 2013; Pages et al., 2009). This is due, in the main, to the exotic pet trade, as large numbers of consignments containing reptiles are imported from the Americas, including species which may act as reservoir hosts. The risk to Europe from RRV had been thought low due to the lack of native marsupials, although feral wallaby populations are known to exist in the UK (Harris and Yalden, 2004). However, the possibility of emergence of RRV in regions without endemic marsupial hosts has been recently suggested (Lau et al., 2017), and RRV has been isolated from trapped male Aedes (Mackenzie et al., 1994), indicating vertical transmission, and meaning that global distribution through drought-resistant eggs may be possible.

8.1.2 Potential for Viral Dissemination and Persistence in the UK

The work described in Chapter 2 demonstrated the potential for equine-mosquito interaction throughout England and showed that vector species, or laboratory competent species of arboviruses affecting horses, are present on a significant proportion of sites. This included the identification of *Aedes vexans*, an important vector species, which is rare in the UK and may be more widely distributed than previously thought.

Container habitats providing breeding sites for *Cx. pipiens* were commonly found on equine premises in Chapter 2, and in Chapter 4 this species was shown to be a potentially efficient vector of JEV at 18 °C. This is significant because 18 °C represents average conditions of a warm summer period (in recent years) in the south of England. As *Cx. pipiens* was not one of the main species collected for this study, short time points were not investigated and therefore the data presented allows for the time taken for *Cx. pipiens* to demonstrate significant transmission to be up to 3 weeks. If this is the case, then transmission is less likely to be ecologically significant at current UK temperatures. However, if significant transmission occurs at 7 days or less at 18 °C, this could have significant implications for European risk assessments and further work to determine EIP at more environmentally temperatures is imperative. JEV has been noted as being of interest in European introduction risk assessments in several studies (de Vos et al., 2017; Durand et al., 2013; Pages et al., 2009), however European mosquito species have only rarely been investigated for competence (de Wispelaere et al., 2017; MacKenzie-Impoinvil et al., 2014).

In Chapter 2 the most commonly found species on equine premises was *Cs. annulata* which is known to be widespread throughout the UK (Medlock et al., 2005), uses a variety of water sources for breeding and was found to be abundant in such sources as muck-heap runoff, although it was also sampled on many sites where the breeding habitat was not identified. In Chapter 5 regular equine-biting by *Cs. annulata* was confirmed, a finding which has been demonstrated previously for European populations (Börstler et al., 2016; Schönenberger et al., 2016). This suggests that for mosquito species with broad host ranges (and without cryptic biotypes, such as *Cx. pipiens molestus*), the likelihood is that host preference is often maintained throughout populations in different countries in Europe. Therefore, where infection risk arises from species-specific host-vector interactions in other European countries, it would be safest to assume that the same would occur in the UK in the event of disease transmission until proven otherwise.

In Chapter 4 the vector competence of *Cs. annulata* for JEV was demonstrated. Vector competence for WNV has been demonstrated previously by colleagues (M. Blagrove,

unpublished). Previous to that work, *Cs. annulata* had only been previously tested for competence for Tahyna virus (Danielova, 1972). However, this species is only found in temperate climes (with little or no transmission of these viruses), so suspicion of potential competence for arboviruses of veterinary or medical import may not have yet occurred. Considering the vector competence results for JEV tested during work for this thesis, it is possible that *Cs. annulata* could be a more efficient vector at lower temperatures than those used here. *Cs. annulata* has been shown to blood feed all year round. The gonotrophic cycle length has been reported to be as short as 5.3 days in June (Service, 1968). Using Davidson's parous method (Davidson, 1954) and parous rates for *Cs. annulata* in the UK (Service, 1968) a rough survival estimate for late summer is that approximately 36% of females will survive (5.8 day gonotrophic cycle length in August) to take a second blood meal, and 21.6% until a third blood meal. Further work to investigate transmission at lower temperatures and shorter time points (less than 14 days) is necessary, therefore, to demonstrate whether *Cs. annulata* could potentially be an ecologically significant vector.

Ochlerotatus detritus was shown in Chapter 2 to be locally abundant on equine premises with reasonable proximity to its particular saltmarsh breeding habitat. This species was shown to regularly travel at least 2.5 km from this habitat to blood feed from horses, in the absence of other livestock. An example of the number of bites which an equine can receive in a single day from *Oc. detritus* in the UK was demonstrated in pilot work included in Chapter 5, where 195 (of 198) blood-fed mosquitoes trapped using one horse, in four hours were *Oc. detritus.* Too little detailed information is known about the dynamics of vector density for this species to comment on how this compares to other seasons, locations or weather conditions. *Oc. detritus* has previously been shown to be a laboratory competent vector of the flaviviruses WNV and JEV (Blagrove et al., 2016; MacKenzie-Impoinvil et al., 2014) and Chapter 4 provides the first evidence of vector competence of *Oc. detritus* for an alphavirus (RRV). Due to the infection dynamics discussed in Chapter 4, describing the efficiency of this species as a vector of RRV is complex: although 50% transmission occurred at 7 days, this dropped subsequently. Gonotrophic cycle length varies throughout the season with the shortest being demonstrated in June (5.4 days) (Service, 1968). Using the methods and sources described above, for *Cs. annulata*, about 36% of *Oc. detritus* females would survive to take a second blood meal. Therefore, a reasonable proportion of those infected could be capable of transmission at this time. It is important to note that RRV is one of the few viruses discussed in this thesis for which there is no vaccine and that there is recent evidence that the risk of global emergence may be higher than previously appreciated (Lau et al., 2017). Future work should include lower temperatures and shorter time points, to

investigate whether significant transmission occurs before 7 days, particularly as an EIP of 5 days has been demonstrated for *Oc. vigilax* at 18 °C (Kay and Jennings, 2002).

In light of the evidence that people may produce viraemia high enough to infect vectors and therefore act as potential transport hosts for VEEV (Morrison et al., 2008) it is reassuring that none of the mosquito species trapped on equine premises were known epizootic vectors of VEEV and both *Cs. annulata* and *Oc. detritus* were inefficient laboratory vectors.

Table 7.1 summarises information regarding potential mosquito vectors of equine arboviruses in the UK.

Species	Host Biting[5,7]	Evidence of Equine Biting	Vector Status
Ae. cinereus / geminus	M[31, 32] B[31, 32]	Morocco[1] Switzerland[32]	EEEV [I][18]
Ae. vexans	M[31, 32] B[31,32]	France[2] Switzerland[32]	WNV [I][5] EEEV [IL"][18,,19,20,21,22,
An. algeriensis	M		
An. claviger	M[32]	Switzerland[32]	
An. maculipennis s.l.	M[31,32] B[31]	UK[4,8] Switzerland[32]	WNV [I][5]
An. plumbeus	M[31]	France[2]	WNV [L][14]
Cq. richiardii	M[31,32] B[32]	France[2] Switzerland[32]	WNV [I][5]
Cs. alaskaensis	M		
Cs. annulata	M[14,32] B[32]	UK[3], France[2] Switzerland[32]	WNV [L][16] JEV [L][3] VEEV [L*][3]
Cs. fumipennis	B		
Cs. litorea	M B		
Cs. longiareolata	B		
Cs. morsitans	M[31] B[31]		EEEV [Z][17, 19]
Cs. subochrea	M[2]	France[2]	
Cx. europaeus	A R B		
Cx. modestus	M[2] B[2]	France[2]	WNV [V L][2.5]
Cx. pipiens s.l.	M[31] B[31,32]	France[2]	WNV [V L][23, 27] JEV [L][23,33,3] EEEV[N][26] WEEV [N][24,25] VEEV [N][27]
Cx. torrentium	B[31] M[31]		
Oc. annulipes	M[9,31] B	France[2]	
Oc. cantans	M[31] B[32]	UK[9] Swizterland[32]	
Oc. caspius	M[2] B[2]	UK[3], France[2]	WNV [I L*][2,5]
Oc. communis	M[31]		
Oc. detritus	M[2] B	UK[3], France[2]	WNV [L][16] JEV [L][16] RRV[L][3] VEEV [L*][3]
Oc. dorsalis	M[6]	UK[6]	WEEV [I L][28,30]
Oc. flavescens	M[11,12]	Denmark, Canada[11, 12]	
Oc. geniculatus	M[2,31]	France[2]	
Oc. leucomelas			
Oc. punctor	M[10] B	UK[10]	WNV [L][14]
Oc. rusticus	M[31, 32] B	Switzerland[32]	
Oc. sticticus	M[31, 32] B[31]	Switzerland[32]	
Or. pulcripalpis	B		

Table 7.1 Mosquito species present in the UK, horse and mammal biting, and vector status for arboviruses of horses

Species underlined were sampled during mosquito samling on equine premises for this thesis.
Information highlighted in red was demonstrated for the first time during work for this thesis.
A- amphibians, B – birds, M – mammals, R – reptiles, L – Laboratory competent vector,
I – Implicated in disease transmission worldwide, N – Non-competent as laboratory vector,
V – Ecologically sign significant bridge vector worldwide, Z – Ecologically significant enzootic vector worldwide,
* -Relatively inefficient laboratory vector, " – Variable laboratory competence in a number of studies.

1. (Faraj et al., 2009)
2. (Balenghien et al., 2006)
3. Work for this thesis
4. (Danabalan, 2010)
5. (Medlock et al., 2005)
6. (Service, 1971b)
7. (Becker et al., 2010)
8. (Hutchinson, 2004)
9. (Medlock and Vaux, 2011)
10. (Service et al., 1986)
11. (Service and Smith, 1972)
12. (Rempel et al., 1946)
13. (MacKenzie-Impoinvil et al., 2014)
14. (Vermeil et al., 1960)
15. (Balenghien et al., 2008)
16. Marcus Blagrove, unpublished observation
17. (Andreadis et al., 1998)
18. (Armstrong and Andreadis, 2010)

19. (Centers for Disease Control and Prevention (CDC), 2006)
20. (Vaidyanathan et al., 1997)
21. (Davis, 1940)
22. (Chamberlain et al., 1954)
23. (Turell et al., 2006)
24. (Aviles et al., 1990)
25. (Hammon and Reeves, 1943)
26. (Merrill et al., 1934a)
27. (Turell, 2012)
28. (Kramer et al., 1998)
29. (Vaux et al., 2015)
30. (Zacks and Paessler, 2010)
31. (Börstler et al., 2016)
32. (Schönenberger et al., 2016)
33. (de Wispelaere et al.,2017)

Only limited virus-vector pairs could be investigated for transmission in this thesis and further work is required. There are still many gaps in our knowledge of the potential for naïve mosquito populations to be vector competent.

Some mosquito species are challenging to test for laboratory competence as they are more difficult to source from the wild, or to colonise. For example, it was not possible to find a source of *Oc. caspius*, even though, in the 2014 season small numbers of this species were obtained by colleagues whilst collecting *Oc. detritus.* It was also not possible to source *An. plumbeus* and *Oc. punctor* was difficult to maintain in captivity and is only available as immatures for a relatively short period in the spring. Virus-mosquito combinations not tested here due to insufficient mosquito numbers, for example, *Cs. annulata* and *Cx. pipiens* for RRV, should also be tested. It is not known whether *Cx. pipiens* form pipiens is vector competent for RRV: Only *Cx. pipiens molestus* has been reported in Australia and neither biotype of *Cx. pipiens* has been competence tested, although RRV has been isolated from *Cx. pipiens molestus* in nature (Russell, 2012). *Cx. pipiens* appears to be uniformly non-competent for the American equine encephalitis viruses (Aviles et al., 1990; Davis, 1940; Hammon and Reeves, 1943; Merrill et al., 1934b; Turell et al., 2003), but is competent for Sindbis virus, which is also an alphavirus (Turell, 2012).

Culiseta species found in the Americas have not been reported as VEEV vectors. However, *Ochlerotatus spp*. such as *Oc. taeniorhynchus* (also laboratory competent), *Oc. sollicitans* and *Oc. thelter*, have been implicated as epizootic vectors of VEEV (Ortiz and Weaver, 2004; Sudia et al., 1975b). Therefore, future work should include vector competence testing of other *Ochlerotatus* species commonly found on equine premises such as *Oc. caspius* and *Oc. punctor*.

EEEV causes fatalities in both horses and humans and therefore Palearctic mosquito species should be tested for vector competence to add to our understanding of the risk of emergence. Other viruses such as WEEV and MVEV are potentially of interest, although WEEV is less pathogenic than EEEV, and MVEV has a limited global distribution.

Although less directly relevant to the aims of this thesis, vector competence testing in Chapter 4 prompted some interesting questions about the possibility of viral clearance by mosquitoes to which a virus is not adapted, and the complexity of relationships between

temperature and transmission. For JEV infection, transmission rates for *Cs. annulata* were significantly reduced at 24 °C compared to 21 °C, and *Oc. detritus* showed apparent clearance of RRV over time. Both of these phenomena have been demonstrated previously but rarely, with different virus-vector pairs (Kay et al., 1989; Reeves et al., 1990; Turell, 1993). Confirmation and further investigation of these phenomena should be carried out as this could inform our understanding of risk associated with naïve mosquito populations, the effects of temperature on mosquito-virus pairs and possibly provide insights into mosquito immunity.

8.1.3 Disease Prevention and Control

SURVEILLANCE

As discussed in Chapter 1, surveillance in the UK is passive and therefore testing of suspect cases in animals or humans is imperative in prompt identification of an outbreak. Disease knowledge of both veterinary surgeons and horse-owners is considered important in early identification in the event of an outbreak of arboviral disease in the UK (Sabirovic et al., 2008a) and whilst the study presented in Chapter 3 demonstrated an awareness of insects such as mosquitoes and biting midges with regards to presence and identification, the majority were unaware of the equine diseases that they transmit. Most study respondents were therefore also unable to provide details of clinical signs and many were not aware of the possibility of fatal outcomes. Education of horse owners about signs of disease will be critical in the event of increased risk of virus transmission in the UK.

PROTECTION OF HORSES

In Chapter 3 most horse-owners stated that they would get advice on fly-bite protection from their veterinary surgeon, at least in the event of an outbreak of disease in the UK. It is therefore imperative that veterinary surgeons should be able to provide accurate information about the most appropriate protection methods, their practicality and efficacy, (or limitations thereof). Prior to this work there have been no specific peer-reviewed, published studies on the efficacy of commercially formulated spray repellent products for the protection of horses from mosquitoes. There is a lack of evidence-based information on how effective prevention methods for mosquito biting of equids are all together. It would, therefore, be difficult for veterinary surgeons to advise clients, and issues such as environmental control and associated regulation may be unknown to UK veterinary surgeons.

The use of spray repellents was the most commonly reported bite prevention method in Chapter 3, and this informed the choice of methods tested in Chapters 5 and 6. Citronella was one of the most commonly used active ingredients as a fly repellent even though it is known to have poor repellent efficacy and cannot be marketed as a repellent for livestock in the EU. This was borne out in the results of the study in Chapter 5 where it was shown to have poor efficacy in protecting horses from mosquito bites. The tested product containing 19.6% DEET was significantly more effective. The results of work conducted for Chapter 6 suggest that products containing 20% icaridin may be even more effective in reducing mosquito biting of horses. Icaridin-based products should be tested in the field to confirm whether icaridin is a more effective repellent than DEET, for horses. However, the practicality of applying spray insect repellents may inhibit their correct use, and they have not been shown in this thesis to provide sufficient protection to prevent arboviral infection under conditions of moderate to high transmission pressure.

With regards to vaccination, respondents in Chapter 3 commonly reported the balance between efficacy and side-effects as a potential barrier to vaccination, as well as the perception of low risk of infection. Recommendation of vaccination (and possibly other prevention) strategies to which horse owners are not accustomed should be done with care, as evidenced by the experience with Hendra virus vaccination in Australia. Based on the results of a questionnaire study it has been suggested that horse owners' reluctance to vaccinate was based on several factors including perceived low risk, but also perception of severe side-effects caused by the vaccine, (although this perception has not been corroborated by veterinary surgeons, nor the regulatory reporting procedures), and the perception that veterinary surgeons are recommending vaccination to make money. It was also apparent that a significant number of horse owners underestimated the severity of disease in humans, and distrusted both the vaccine company and the motives of the veterinary profession in recommending vaccination, despite horse-handlers and veterinary surgeons having died from contracting Hendra virus from horses, and release of the vaccine being expedited to save lives (Goyen et al., 2017). In the event of increased risk to the UK, it would be vitally important that policy makers develop guidance for protection of horses (and humans) from infection and encourage vaccine companies to apply for licenses for appropriate vaccines in the UK.

Further work should include investigating the current knowledge of UK veterinary surgeons, regarding arboviral diseases, and effective methods for disseminating information regarding surveillance, prevention and increase in risk to the veterinary profession, equine industry and related stakeholders.

Conclusions

This thesis has provided novel data about the presence of vector mosquito species and potential vector species on equine premises in the UK and has added to the data for regional distributions of these species. Where risk has been demonstrated arising from host-vector interaction between two species in other European countries, it is safest to assume the same could occur in the UK, until specific research in the UK demonstrates otherwise. However, significant outbreak risk is likely to be contingent on an increase in mosquito density with climate change, and a concomitant increase in host-vector interaction.

This work has added to the numbers of laboratory competent vector species of JEV known in Europe and provides the first demonstration of the ability of *Oc. detritus* to transmit an alphavirus. WNV is known to have a broad host and vector range and the vector competence results, presented here, show that viruses other than WNV may have wider potential vector ranges than previously appreciated. Although the risk of autochthonous transmission at current UK temperatures may be relatively low, the evidence provided here emphasises the importance of further work on transmission of arboviruses at lower temperatures.

Horse-owner knowledge of insect-borne disease and prevention methods in the UK is poor and this study has shown that individual protection methods such as spray repellents are unlikely to be adequate in preventing arboviral infection. However this work as provided evidence that they have some utility in the case of low transmission pressure for viruses for which vaccines are not available.

Whilst the current risk to UK horses is likely to be low, how long it remains so, is difficult to predict. Further work on the vector competence of native mosquitoes and protection methods specific to UK conditions is required to inform risk prediction.

In the event of indicators of increased risk to the UK (such as emerging disease, or increased transmission in continental Europe) policy makers should give serious consideration to increased surveillance for arbovirus circulation in the UK, particularly as these viruses cause significant disease in humans as well as horses. This should be allied with encouraging vaccine companies to prepare for equine vaccine licensing in the UK, where vaccines are available, and educating veterinary surgeons and horse owners regarding clinical signs of disease and appropriate prevention methods.

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9 APPENDICES

Appendix A

COI sequences for 3 adult *Aedes vexans* trapped during fieldwork for Chapter 2. (Table 2.2**) *courtesy of Luis Hernandez-Triana, APHA, Addlestone, Surrey.***

>CXMDO058-17|APHA-13-2017E10|Aedes_vexans|COI-5P

AACATTATATTTTATTTTTGGAGTTTGATCAGGAATAGTAGGAACATCTTTAAGT
ATATTAATTCGTGCTGAATTAAGACACCCAGGGATATTTATTGGAAATGATCAA
ATTTATAACGTAATTGTTACAGCTCATGCATTTATTATAATTTTTTTTATAGTAAT
ACCAATTATAATTGGAGGATTCGGAAATTGATTAGTTCCTTTAATATTAGGAGCT
CCTGATATAGCTTTTCCTCGAATAAATAATATAAGTTTTTGAATATTACCTCCTT
CATTAACTCTACTACTTTCTAGTTCAATAGTAGAAAATGGAGCAGGGACAGGAT
GAACAGTTTATCCTCCTCTTTCATCAGGAACAGCTCACGCTGGGGCTTCAGTTGA
TTTAGCTATTTTCTCTCTTCATTTAGCTGGGATTTCATCTATTTTAGGGGCAGTAA
ATTTTATTACTACAGTTATTAATATACGATCATCTGGAATTACTTTAGATCGATT
ACCTTTATTTGTTTGATCAGTAGTAATTACTGCTATTTTATTACTTTTATCTCTTC
CCGTATTAGCTGGAGCTATTACTATATTATTAACTGATCGAAATTTAAATACTTC
ATTCTTCGATCCAATTGGAGGAGGAGATCCTATTCTTTATCAACATTTATTT

>CXMDO059-17|APHA-13-2017E11|Aedes_vexans|COI-5P

AACATTATATTTTATTTTTGGAGTTTGATCAGGAATAGTAGGAACATCTTTAAGT
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ACCAATTATAATTGGAGGATTCGGAAATTGATTAGTTCCTTTAATATTAGGAGCT
CCTGATATAGCTTTTCCTCGAATAAATAATATAAGTTTTTGAATATTACCTCCTT
CATTAACTCTACTACTTTCTAGTTCAATAGTAGAAAATGGAGCAGGGACAGGAT
GAACAGTTTATCCTCCTCTTTCATCAGGAACAGCTCACGCTGGGGCTTCAGTTGA
TTTAGCTATTTTCTCTCTTCATTTAGCTGGGATTTCATCTATTTTAGGGGCAGTAA
ATTTTATTACTACAGTTATTAATATACGATCATCTGGAATTACTTTAGATCGATT
ACCTTTATTTGTTTGATCAGTAGTAATTACTGCTATTTTATTACTTTTATCTCTTC
CCGTATTAGCTGGAGCTATTACTATATTATTAACTGATCGAAATTTAAATACTTC
ATTCTTCGATCCAATTGGAGGAGGAGATCCTATTCTTTATCAACATTTATTT

>CXMDO060-17|APHA-13-2017E12|Aedes_vexans|COI-5P

AACATTATATTTTATTTTTGGAGTTTGATCAGGAATAGTAGGAACATCTTTAAGT
ATATTAATTCGTGCTGAATTAAGACACCCAGGGATATTTATTGGAAATGATCAA
ATTTATAACGTAATTGTTACAGCTCATGCATTTATTATAATTTTTTTTATAGTAAT
ACCAATTATAATTGGAGGATTCGGAAATTGATTAGTTCCTTTAATATTAGGAGCT
CCTGATATAGCTTTTCCTCGAATAAATAATATAAGTTTTTGAATATTACCTCCTT
CATTAACTCTACTACTTTCTAGTTCAATAGTAGAAAATGGAGCAGGGACAGGAT
GAACAGTTTATCCTCCTCTTTCATCAGGAACAGCTCACGCTGGGGCTTCAGTTGA
TTTAGCTATTTTCTCTCTTCATTTAGCTGGGATTTCATCTATTTTAGGAGCAGTAA
ATTTTATTACTACAGTTATTAATATACGATCATCTGGAATTACTTTAGATCGATT
ACCTTTATTTGTTTGATCAGTAGTAATTACTGCTATTTTATTACTTTTATCTCTTC
CTGTATTAGCTGGAGCTATTACTATATTATTAACTGATCGAAATTTAAATACTTC
ATTCTTCGATCCAATTGGAGGAGGAGATCCTATTCTTTATCAACATTTATTT

Appendix B (Chapman et al. 2016)

Paper

OPEN ACCESS

Potential vectors of equine arboviruses in the UK

G. E. Chapman, D. Archer, S. Torr, T. Solomon, M. Baylis

There is growing concern about the increasing risk of disease outbreaks caused by arthropod-borne viruses (arboviruses) in both human beings and animals. There are several mosquito-borne viral diseases that cause varying levels of morbidity and mortality in horses and that can have substantial welfare and economic ramifications. While none has been recorded in the UK, vector species for some of these viruses are present, suggesting that UK equines may be at risk. The authors undertook, therefore, the first study of mosquito species on equine premises in the UK. Mosquito magnet traps and red-box traps were used to sample adults, and larvae were collected from water sources such as tyres, buckets, ditches and pools. Several species that are known to be capable of transmitting important equine infectious arboviruses were trapped. The most abundant, with a maximum catch of 173 in 72 hours, was *Ochlerotatus detritus*, a competent vector of some flaviviruses; the highest densities were found near saltmarsh habitats. The most widespread species, recorded at >75 per cent of sites, was *Culiseta annulata*. This study demonstrates that potential mosquito vectors of arboviruses, including those known to be capable of infecting horses, are present and may be abundant on equine premises in the UK.

Globally, there is increasing concern over emerging infectious diseases, particularly arthropod-borne viruses (arboviruses) affecting human beings and livestock (Kilpatrick and Randolph 2012, Durand and others 2013). Examples from the UK include bluetongue and Schmallenberg viruses in ruminants. The introduction of West Nile virus (WNV) into North America demonstrated the effects of mosquito-borne disease on a naive host population, both human and equine, and concerns have also been raised over the potential for spread of other mosquito-borne arboviruses affecting horses (Brown and others 2008, Pages and others 2009, Durand and others 2013). Mosquito-borne arboviruses affecting horses include WNV, Japanese encephalitis virus (JEV), Eastern equine encephalitis virus (EEEV), Western equine encephalitis virus (WEEV), Venezuelan equine encephalitis virus (VEEV), Ross River virus (RRV), Murray Valley encephalitis virus (MVEV) and Getah virus (Table 1).

Further knowledge about potential vector mosquitoes in the UK and their ability to spread arboviruses will play a key role in control and surveillance of disease in the event of an outbreak. Climate change may increase the risk of emergence of arboviral diseases by several mechanisms. Higher temperatures increase the ability of vectors to transmit viruses (Guis and others 2012, MacKenzie-Impoinvil and others 2015) and also have the potential to increase the geographical range of mosquitoes (Elbers and others 2015). Increased winter rainfall may also contribute to increase in mosquito populations due to creation of more temporary freshwater sites for breeding and greater abundance of emerging mosquitoes in spring (Vardoulakis and Heaviside 2012).

There are 34 species of mosquito in the UK (Medlock and Vaux 2011) and species that are implicated as vectors of arboviruses of horses elsewhere in the world include *Aedes cinereus, Aedes vexans, Anopheles maculipennis s.l., Coquillettidia richiardii, Culex pipiens, Culex modestus, Culiseta morsitans, Ochlerotatus caspius, Ochlerotatus dorsalis and Ochlerotatus flavescens* and a number of these are widely distributed and locally abundant across the UK (Table 2). In addition, some mosquito species present in the UK have been shown in the laboratory to be competent vectors of at least one of these viruses including *Ochlerotatus punctor, Oc. detritus, Cx. modestus, Ae. vexans, Cx. pipiens s.l.* and *Anopheles plumbeus* (Table 2).

In the UK, there have been recent and ongoing sampling and surveillance of mosquito species (Snow and Medlock 2008, Medlock and Vaux 2013, 2014, 2015, Vaux and Medlock 2015, Vaux and others 2015); however, there has been no sampling of mosquito species with specific focus on the equine host. Accordingly, the authors carried out a survey of the mosquitoes present at 32 premises across England (see Fig 1 for approximate locations) to obtain baseline data on the species composition and abundance of mosquitoes that may interact readily with equines. The authors' results identify which species may play an important role in outbreaks of mosquito-borne equine viruses in the UK and hence contribute to the development of national strategies to monitor and manage this risk.

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Methods

A total of 32 sites were sampled—8 equine premises in each of northwest, northeast, southeast and southwest regions in

TABLE 1: Mosquito-borne viruses affecting horses and known morbidity and mortality information

	Virus							
	JEV	WNV	EEEV	WEEV	VEEV	MVEV	RRV	Getah virus
Inapparent infections common	Yes[9]	Yes[7]	Yes[2]	Yes	No[9]	Yes[12]	Yes[12]	Yes
Morbidity	0.03-1.4% of horses in a region[3]	1 in 11-12 infections[7]	61%[1] of horses on some farms	low	10% of regional population (estimated)[10,11]	Low	Low	Unknown
Case mortality	5-40%[4,5,6]	38-57%[7]	Up to 73%[1]	20-30%[8]	40-90%[10,11]	Low	Low	Not fatal
Vaccination available	Y	UK licensed	Y	Y	Y			Y

Y—available in affected countries
[1]Silva and others (2011)
[2]Pauvolid-Corrêa and others (2010)
[3]Spickler (2010)
[4]Ellis and others (2000)
[5]Hale and Withington (1953)
[6]Nakamura (1972)
[7]Sellon and Long (2013)
[8]Long and Gibbs (2007)
[9]Rico-Hesse (2000)
[10]Sudia and others (1975)
[11]Zehmer and others (1974)
[12]Vale and others (1991)
[13]Holmes and others (2012)
EEEV, Eastern equine encephalitis virus; JEV, Japanese encephalitis virus; MVEV, Murray Valley encephalitis virus; RRV, Ross River virus; VEEV, Venezuelan equine encephalitis virus; WEEV, Western equine encephalitis virus; WNV, West Nile virus

England (Fig 1). A stratified sampling approach was used due to the fact that many mosquito species have a patchy population distribution. In this case, four types of mosquito breeding habitat were identified: land associated with drainage ditches (drained farmland) or fenland (site 29), woodland, urban and salt marsh (Hutchinson and others 2007). The authors aimed to recruit two equine premises in each of the four habitats in each region (32 premises in total).

An internet search was conducted using Google Maps and The Phone Book from British Telecom, using the search terms 'Riding Schools', 'Livery', 'Stables', 'Stud'. BHS Riding Schools and Livery Yard Lists and the British Equestrian Directory and Newmarket Trainers Association lists were also used. This produced a list of businesses with publicly available contact details.

For each premises, the local area was investigated for potential mosquito habitats using Magic (www.magic.gov.uk) and Google Earth. Sites were assigned a category based on habitat (some sites qualified for two categories) and were graded based on area of presumed habitat and proximity of habitat to the premises. The authors aimed to locate premises within suitable habitats or, if that was not possible, within 500 m (woodland and urban sites), 1 km (urban and drained farmland sites) or 3 km (salt marsh). A maximum distance of 500 m for woodland sites was selected reflecting the relative ease of finding sites close to woodland. For salt marsh or grazing marsh, it was not possible to find sites in close proximity in many cases, but species associated with floodwater, such as *Ae. vexans* and coastal salt marsh such as *Oc. detritus*, tend to have greater dispersal capacity and *Oc. detritus* is capable of flying at least 2.5 miles (Service 1969, 1971b, Snow and Medlock 2008, Becker and others 2010, Verdonschot and Besse-Lototskaya 2014). In order to try and include all four habitat types within reasonable travelling distance, the four areas within the regions were chosen as follows: Wirral peninsula and Chester (northwest); between Scunthorpe, Gainsborough, Doncaster and Goole (northeast); within 20 miles of Exeter (southwest); a transect from Newmarket to the Wash (southeast).

Premises were recruited by sending out either a letter or email to the business, and following up with a telephone call. For sites where there was no response or a negative response, correspondence was then sent to a number of alternative second-choice sites, for that category of habitat, until 32 sites (eight in each of four regions) were recruited.

Mosquito sampling

Host-seeking adults

Each of the 32 sites was visited three times throughout the summer of 2015, and mosquitoes were trapped continuously for three days. Timing of visits was based on what is presently known about peaks in adult mosquito numbers of different species in the UK, visiting each of four regions within each of three seasonal peaks of mosquito activity in the months of May, late June–early July and September (Service 1969, 1977, Medlock and others 2007, Snow and Medlock 2008, Becker and others 2010, Medlock and Vaux 2015).

Trapping at each site was carried out using a mosquito magnet, Independence model (Woodstream Europe). The mosquito magnet is designed to catch host-seeking mosquitoes by using propane as a fuel source to produce heat, moisture and carbon dioxide. The trap was also baited with 1-octen-3-ol (as supplied by the trap manufacturer). The mosquito magnet trap was run continuously for ~72 hours starting in the morning and a data logger was placed underneath the body of the trap to record the environmental temperature and relative humidity for this time period. The traps were emptied as time allowed, so that the samples were not in the trap for more than 48 hours, to reduce damage, and the subsamples were combined and identified as the full 72-hour catch.

Attempts were made to catch mosquitoes landing on hosts in order to confirm horse biting. These attempts were made in the afternoon and wherever possible at dusk (Service 1969, 1971a). To fit in with the trapping schedule, four sites in each area were sampled in June/July and September in the mid-late afternoon, and four sites around dusk. Therefore, one site per day was sampled in the afternoon and one in the evening for Monday to Thursday of the trapping week in each sampling area. For each sampling effort, a group of horses was observed for 15 minutes to see whether any mosquitoes could be identified landing on them. If no mosquitoes were observed, then another group of horses was observed for 15 minutes. Group sizes ranged from 1 to 10 as horses were in their normal grazing environment (with the exception of site 25 where sampling was attempted in the stable as there was no grazing). If no mosquitoes were observed on two groups, the attempt was abandoned. If mosquitoes were observed, landings were counted for 2 minutes and then mosquitoes sampled from the head and neck of the horse (for reasons of safety) for 30 minutes to allow for species identification. Some premises could not be sampled at dusk due to access restrictions, so were only sampled in the

TABLE 2: Mosquito species present in the UK, horse and mammal biting, and vector status for arboviruses of horses

Species	Host biting[5,7]	Evidence of equine biting	Vector status
Aedes cinereus/Aedes geminus	M[21, 32] B[21, 32]	Morocco[1], Switzerland[32]	EEE V (l)[35]
Aedes vexans	M[21, 32], B[21, 32]	**France[2], Switzerland[32]**	**WNV (l)[6], EEV (l, L*)[18,19,20,21,22],**
Anopheles algeriensis	M		
Anopheles claviger	M[32]	**Switzerland[32]**	
Anopheles maculipennis s.l.	M[21,32], B[21]	**UK[4,8], Switzerland[32]**	**WNV (l)[6]**
Anopheles plumbeus	M[21]	**France[2]**	**WNV (L)[34]**
Coquillettidia richiardii	M[21,32], B[32]	**France[2], Switzerland[32]**	**WNV (l)[6]**
Culiseta alaskaensis	M		
Culiseta annulata	M[14,32], B[32]	**UK[8], France[2], Switzerland[32]**	**WNV (L)[33]**
Culiseta fumipennis	B		
Culiseta litorea	M, B		
Culiseta longiareolata	B		
Culiseta morsitans	M[21], B[21]		**EEV (Z)[17, 19]**
Culiseta subochrea	M[2]	**France[2]**	
Culex europaeus	A, R, B		
Culex modestus	M[2], B[2]	France[2]	WNV (V, L)[2,5]
Culex pipiens s.l.	M[21] B[14,32]	**France[2]**	**WNV (V, L)[23, 27], JEV (L†)[23], EEV (N)[26], WEEV (N)[24,25], VEEV (N)[27]**
Culex torrentium	B[21], M[21]		
Ochlerotatus annulipes	M[9,21], B	France[2]	
Ochlerotatus cantans	M[21], B[32]	**UK[8], Switzerland[32]**	
Ochlerotatus caspius	M[2], B[2]	**UK[8], France[2]**	**WNV (l, L†)[2,5]**
Ochlerotatus communis	M[21]		
Ochlerotatus detritus	M[2], B	**UK[8], France[2]**	**WNV (L)[34], JEV (L)[33]**
Ochlerotatus dorsalis	M[8]	**UK[8]**	**WEEV (l, L)[28,29]**
Ochlerotatus flavescens	M[11, 12]	Denmark, Canada[11, 12]	
Ochlerotatus geniculatus	M[2,21]	France[2]	
Ochlerotatus leucomelas			
Ochlerotatus punctor	M[10], B	**UK[10]**	**WNV (L)[34]**
Ochlerotatus rusticus	M[21, 32], B	**Switzerland[32]**	
Ochlerotatus sticticus	M[21, 32], B[21]	Switzerland[32]	
Orthopodomyia pulcripalpis	B		

Species in bold were sampled during the present study
*Variable laboratory competence in a number of studies
†Relatively inefficient laboratory vector
[1]Faraj and others (2009)
[2]Balenghien and others (2006)
[3]Pilot work for this study—site 8, 2014
[4]Danabalan (2010)
[5]Medlock and others (2005)
[6]Service (1971a)
[7]Becker and others (2010)
[8]Hutchinson (2004)
[9]Medlock and Vaux (2011)
[10]Service and others (1986)
[11]Service and Smith (1972)
[12]Rempel and others (1946)
[13]MacKenzie-Impoinvil and others (2015)
[14]Vermeil and others (1960)
[15]Balenghien and others (2008)
[16]Blagrove and others, (2016)
[17]Andreadis and others (1998)
[18]Armstrong and Andreadis (2010)
[19]Centers for Disease Control and Prevention (CDC) (2006)
[20]Vaidyanathan and others (1997)
[21]Davis (1940)
[22]Chamberlain and others (1954)
[23]Turell and others (2006)
[24]Aviles and others (1990)
[25]Hammon and Reeves (1943)
[26]Merrill and others (1934)
[27]Turell (2012)
[28]Kramer and others (1998)
[29]Vaux and others (2015)
[30]Zacks and Paessler (2010)
[31]Börstler and others (2016)
[32]Schönenberger and others (2016)
[33]M.S.C. Blagrove, personal communication
A, amphibians; B, birds; EEEV, Eastern equine encephalitis virus; I, implicated in disease transmission worldwide; JEV, Japanese encephalitis virus; L, laboratory-competent vector; M, mammals; N, non-competent as laboratory vector; R, reptiles; V, ecologically significant bridge vector worldwide; VEEV, Venezuelan equine encephalitis virus; WEEV, Western equine encephalitis virus; WNV, West Nile virus; Z, ecologically significant enzootic vector worldwide

afternoon. In order to trap mosquitoes feeding on horses, a mechanical pooter (Watkins and Doncaster) was modified with an elongated inlet tube and was muffled so as to avoid startling the horse. Individual horse behaviour was discussed with the yard owner in advance, and permission to attempt landing catches with each horse or group of horses was obtained.

Resting adults

The resting box trap was a 40×30×20 cm black box (Morris 1981), painted red inside (red-box trap) and was designed to aid in the capture of blood-fed mosquitoes (Fig 2). It was set in an open area facing west and was emptied on two mornings (either at 24 and 72 hours after deployment or 48 and 72 hours) by

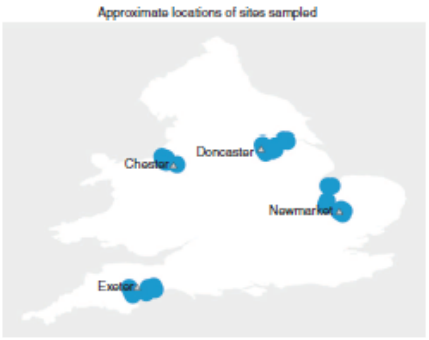

FIG 1: Map of study areas

placing a perspex cover on the open front of the box and aspirating resting mosquitoes.

Immature mosquitoes

Larval sampling was undertaken on the equine premises themselves and, where there was access, on neighbouring land within 500 m of the mosquito magnet or of grazing horses. The aim was to sample all water sources within the boundary of the premises, including all collections of artificial containers. This was not always possible due to access constraints or on larger premises. Larvae and pupae were sampled using a dipper. This is a 500 ml container with a long handle that is used to sample water sources. Each dip was then emptied into a white tray and searched for larvae. For larger water bodies, 5×500 ml dips were used in different parts of the water body, whereas for small containers only one dip sample or partial dip samples could be obtained.

Sample handling and identification

Mosquitoes were removed from the traps with a mechanical aspirator and 'Fly-nap' (Carolina Biological Supply Company) was used to produce knockdown. Adult mosquitoes were stored dry and identified within four days. Blood-fed mosquitoes were stored in 90 per cent ethanol immediately.

Larvae were pipetted into universal containers for storage. Fourth instar larvae were killed by gradually adding 90 per cent ethanol. Pupae were allowed to emerge for ease of identification.

FIG 2: Red-box trap

Live second and third instar larvae were allowed to continue to develop until the end of the fieldwork week for ease of identification. Containers were inspected daily and any dead larvae or pupae were preserved using 90 per cent ethanol for identification (Snow 1991).

Mosquitoes of all stages were identified morphologically as far as possible to species or species complex using keys of British and European mosquitoes (Marshall 1938, Cranston and others 1987, Snow 1991, Schaffner and others 2001, Becker and others 2010). *Cx. pipiens* was differentiated from *Culex torrentium* by molecular methods as described by Hesson and others (2010).

Due to the skewed distribution of the catch data, geometric mean is used for comparison in the text and Fig 3. Fig 4, showing total catch counts, is displayed with a log(10) scale.

Results

Host-seeking adults

It was not possible to find drained farmland in the southwest area sampled, so two more exposed hillside sites were chosen as a comparison (sites 18 and 19, at altitudes of 120 and 114 m, respectively). At one of these hillside locations (location 19, Table 3), trapping was not carried out in September 2015 due to loss of the propane canister. A number of specimens could not be identified positively to species level due to trap damage and are recorded as unidentified *Aedes* species.

A total of 917 adult mosquitoes of 14 species were caught over a total of 285 trapping days over the 32 locations (Table 3). The geometric mean catch for each mosquito magnet trapping period (approximately 72 hours) was 3.7 (sd 3.4), across all locations and seasons. Totals caught were 487, 217, 160 and 53 in the areas sampled in the northwest, southeast, northeast and southwest, respectively.

For locations given one habitat classification, the geometric mean catch (nine days across three sampling periods) from a mosquito magnet was 6.9 (sd 5.90), 3.8 (2.5), 6.1 (3.3) and 36.5 (5.2) for premises associated with woodland, urban, drained farmland and saltmarsh habitats, respectively (Fig 3).

The most abundantly trapped species was *Oc. detritus* with a total of 499 adults caught. All three sites with total catches >100 were associated with the saltmarsh habitat of this species.

The second most abundantly trapped species was *Cs. annulata*, with 154 adults caught. *Cs. annulata* had the highest presence and was trapped on 75 per cent (24/32) of sites.

Total catch was highest in September (Fig 4), and the difference in catch was significantly higher ($P<0.005$) than that in May and that in June/July in a general linear model with a

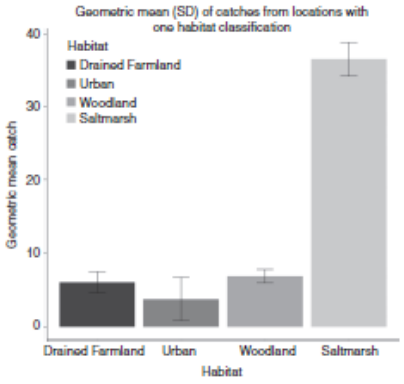

FIG 3: Geometric mean of total catch per location for each habitat type (locations only included if given one habitat)

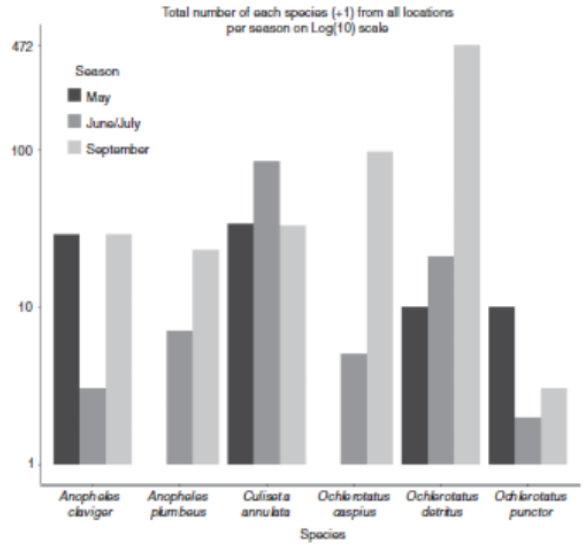

FIG 4: Total adult catches by season for each of six most abundant species

TABLE 3: Adult mosquito species and number trapped in mosquito magnet trap

Location number and region	Habitats	Most abundantly caught mosquito species						Other	Total
		Anopheles claviger	*Anopheles plumbeus*	*Culiseta annulata*	*Ochlerotatus caspius*	*Ochlerotatus detritus*	*Ochlerotatus punctor*		
NW 1	D	0	0	12	0	0	0		12
NW 2	U	6	0	7	24	1	0	*UA*—5	43
NW 3	D	5	0	0	0	0	0		5
NW 4	U, S	0	1	12	3	53	0	*UA*—5	74
NW 5	W	0	1	5	0	2	0	*OCA*—3	11
NW 6	W	0	0	8	3	17	0		28
NW 7	S	0	0	14	1	176	0	*UA*—4	195
NW 8	W, S	3	11	12	4	85	0	*UA*—4	119
NW 9	U	0	0	1	0	0	0		1
NW 10	W, D	16	0	15	2	0	10	*OCA*—3, *CR*—2, *UA*-9 *AV*—3	60
NE 11	W	0	0	6	0	0	0		6
NE 12	W, U	1	0	20	0	0	0	*CuS*—1, *CR*—1	23
NE 13	S	8	0	2	19	0	0		29
NE 14	S	5	0	0	3	1	0	*CR*—3	12
NE 15	D	6	0	15	0	0	0	*AnM*—1, *CR*—1 *UA*—1	24
NE 16	U	3	0	2	0	0	0		5
SW 17	W	1	0	2	0	0	0	*AnM*—1	4
SW 18	H	0	0	0	0	0	0		0
SW 19	H	0	0	0	0	0	0	*CaP*—1	1
SW 20	W, S	3	0	2	0	4	0	*CaP*—1	10
SW 21	S, U	0	1	0	8	0	0	*CaP*—1, *UA*—8	18
SW 22	W, U	0	0	0	0	1	2		3
SW 23	W	0	13	1	0	0	0		14
SW 24	W	0	1	1	0	0	0	*CaP*—1	3
SE 25	U	0	0	0	0	0	0	*CaP*—1	1
SE 26	W	0	0	1	0	0	0	*OD*—1	2
SE 27	W, U	0	0	0	0	0	0	*CR*—1	1
SE 28	W	0	0	6	0	0	0	*CR*—3	9
SE 29	D	0	0	3	1	0	0	*CR*—2	6
SE 30	S	0	0	4	33	155	0		192
SE 31	S	1	0	2	0	2	0		5
SE 32	D	0	0	1	0	0	0		1

AnM, An. maculipennis; AV, Ae. vexans; CR, Cq. richiardii; CuS, Cs. subochrea; CaP, Cx. pipiens s.l.; D, drained farmland; NE, northeast; NW, northwest; *OCA, Oc. cantans; OD, Oc. dorsalis; OR, Oc. rusticus*; S, salt marsh; SE, southeast; SW, southwest; U, urban; *UA*, unidentified *Aedes* species; W, woodland

negative binomial distribution using the R statistical programme and the MASS package (The R Foundation (2016) R: The R Project for Statistical Computing. The R Foundation. https://www.r-project.org/. Accessed June 15, 2016; Ripley and others 2016). Thirty-one sites were sampled (one site not sampled), and total mosquito number from all locations was 679 with a geometric mean of 5.6 (sd 5.1) per location.

No mosquitoes were trapped while feeding on horses. Only three blood-fed mosquitoes were trapped, all were part-fed individuals caught in the mosquito magnet, of which two were *Oc. detritus* and one was *Cs. annulata*. One mosquito (*Oc. caspius*) was sampled landing on a human host. Pilot host-landing catches using horses carried out in September 2014 yielded 20 *Oc. detritus*, 3 *Oc. caspius* and 2 *Cs. annulata*, in two 15-minute daytime sampling efforts at site 8.

Resting adults

Sampling of resting mosquitoes was unsuccessful. No mosquitoes were found in the red-box traps.

Immature mosquitoes

Immature mosquitoes were recovered by dipping of water sources on 23 of 32 premises. A total of 61 samples containing mosquito larvae or pupae were collected from a variety of water sources including ditches, buckets and water butts, tyres, ruts, muck heaps, pools and ponds.

Cx. pipiens s.l., *Cx. torrentium*, *Cs. annulata/alaskaensis/subochrea*, *Culiseta fumipennis*, *Cs. morsitans*, *Oc. caspius*, *Anopheles claviger* and *An. maculipennis s.l.* were captured using dipping techniques.

The majority of samples were from artificial containers with small amounts of water, such as tyres. Therefore on most occasions, samples from each container were less than 500 ml, so it was not considered appropriate to state the numbers sampled, nor possible to compare larval numbers across sites. Larval samples were used to identify the presence of a species rather than its relative abundance.

A selection of larvae identified morphologically as *Cx. pipiens/torrentium* was further identified by molecular methods for each location. Of the 23 sites from which samples were obtained, *Cx. pipiens* larvae were identified from 65.2 per cent of locations, *Cx. torrentium* from 47.8 per cent. Both species were found on 21.7 per cent of these 23 locations. Both *Cx. pipiens* and *Cx. torrentium* larvae were obtained from at least two sites in all four regions.

Cs. annulata/alaskaensis/subochrea larvae cannot be differentiated morphologically and were obtained at 28.1 per cent (9/32) of sites. Due to the rarity of *Cs. alaskaensis* and the relative abundance of *Cs. annulata*, it is likely that the vast majority of these were *Cs. annulata*. The total of sites with presence of *Cs. annulata/alaskaensis/subochrea* including adult samples was 84.4 per cent (27/32).

Discussion

This study is, to the authors' knowledge, the first survey of mosquito species on equine premises in the UK. This work has demonstrated the presence of several mosquito species that are candidate vectors of pathogens affecting horses. Commonly found mosquito species on equine premises during this study included *Oc. detritus*, *Oc. caspius*, *Cs. annulata*, *Cx. pipiens s.l.*, *Cx. torrentium*, *An. claviger*, *An. plumbeus* and *Oc. punctor*. Although mosquito density could be considered low at most of the sites sampled, this could be partly explained by the fact that the months of March, April and May in 2015 were relatively dry for all of the regions except the northwest (Met Office 2016). Climate change predictions suggest increased temperature and potential for flooding events in the UK (Met Office 2010, Caminade and others 2012, Medlock and Leach 2015), which are likely to increase the abundance of native mosquito species. It therefore seems likely that in the future there may be significantly increased horse–vector interaction, particularly with mosquito species that thrive in warmer regions of Europe, such as *Cx. annulata*, *Oc. caspius*, *Cx. pipiens s.l.* *Oc. detritus*, *An. plumbeus*, *Cq. richiardii*, *An. maculipennis and Ae. vexans* (Balenghien and others 2008). The species trapped in the current study are all considered mammalophilic or bite both birds and mammals, with the exception of *Cx. torrentium* that is strongly ornithophilic (bird biting). Three European studies provide evidence that *Cx. pipiens s.l.* found in rural areas will bite mammals, including horses (Balenghien and others 2008, Börstler and others 2016, Schönenberger and others 2016). Although not all of these studies differentiated *Cx. pipiens* form *pipiens* from *Cx. pipiens* form *molestus*, the study of Börstler and others (2016) records a significant number of *Cx. pipiens* form *pipiens* with mammalian blood meals. *Oc. detritus*, *Oc. caspius*, *Cx. pipiens s.l.*, *Oc. punctor* and *An. plumbeus* have all been shown to transmit arboviruses affecting horses and therefore are important when considering the risk of mosquito-borne equine disease. Also, 11 of the 16 species found on equine premises during this study are laboratory-competent vectors of, or are implicated in, naturally occurring disease cycles for at least one arbovirus-affecting horses (Table 2). An important aspect of this study is that the authors trapped very few blood-fed mosquitoes: just three in the mosquito magnet and none by other methods. This begs the question of whether the mosquitoes present at equine premises in the UK only rarely feed on equines or whether they feed but were not caught. A number of factors suggest that the latter is the most likely explanation: (i) the mosquito magnet is designed to trap host-seeking rather than blood-fed adults; (ii) many of the premises had other potential hosts present (human beings, cattle, small mammals), indicating that the low number of trapped blood-fed mosquitoes cannot be attributed to the specific avoidance of equids; (iii) in pilot work in September 2014, mosquitoes *Cs. annulata*, *Oc. caspius and Oc. detritus* were directly observed by the author feeding on horses; and (iv) most of the species caught in this study have been reported, in other studies, to feed on horses and/or transmit arboviruses to horses. Nevertheless, and probably due to the inherent difficulties in trapping blood-fed mosquitoes in the UK (Brugman and others 2015), blood-feeding on horses has not been confirmed in this study. A large sampling effort and high mosquito densities are required to maximise trapping of blood-fed mosquitoes. The number of sites included in this study dictated that sampling effort on each site was necessarily lower than that of other recent studies (Brugman 2016), and seasonal variation in abundance due to climatic conditions, for example, a dry early summer period (Met Office 2016), may have supressed mosquito density. However, all of the species sampled in this study, with the exception of *Cx. torrentium* and *Cs. morsitans*, have been shown to bite equines (Table 2), and four of the six most abundant species in adult catches have been shown to bite horses in the UK either in previous studies or in pilot work for this study. Further work would be required to investigate the feeding rate of UK populations of these mosquitoes on horses, and host bait catches (Schönenberger and others 2016) would seem most likely to provide useful information.

The comparatively high numbers of *Oc. detritus* and *Oc. caspius* caught on some saltmarsh-associated sites are consistent with previous studies and reports of significant nuisance biting (Clarkson and Setzkorn 2011, Medlock and others 2012, Medlock and Vaux 2013) and confirm that there is significant potential for host–vector interaction between these species and horses. These two species are competent vectors of WNV (Vermeil and others 1960, Blagrove and others, 2016). Detailed, high resolution information regarding horse and mosquito species distribution is lacking (Lo Iacono and others 2013). However, using previously published horse distribution data at postcode scale (Boden and others 2012, Lo Iacono and others 2013) and saltmarsh distribution (Adnitt and others 2007), in combination with mosquito species records, several coastal areas of England appear worthy of further investigation for host–vector interaction potential. These areas have high horse density, saltmarsh presence and records of *Oc. detritus* and *Oc. caspius*

(The Walter Reed Biosystematics Unit 2014, National Biodiversity Network 2016a, b) and include the Severn estuary, South Devon coast, the south coast of England from Swanage to Chichester and the Dee and Mersey estuaries. Two of these areas were sampled during this study: Wirral (Dee estuary) and the South Devon coast.

The finding that the WNV vector *Cx. pipiens* was common on equine premises with suitable water sources is expected as this species has a widespread distribution (Medlock and others 2005, Medlock and Vaux 2011), but this study confirms that suitable container habitats are commonplace on equine premises. *Cx. torrentium* is a major enzootic (wildlife) vector of Sindbis virus in Scandinavia (Hesson and others 2015) and may therefore be capable of a similar role in transmission of other arboviruses. *Cx. pipiens* and *Cx. torrentium* were found on a number of occasions in all four regions, suggesting that *Cx. torrentium* may be more prevalent in the north of England than previously recognised (Medlock and others 2005).

One of the most interesting results to emerge from the current study was the presence of *Cs. annulata* on the majority of sites (27/32). It was also the second most abundant species in mosquito magnet samples, after *Oc. detritus*. While *Cs. annulata* is known to have a widespread distribution in the UK (Medlock and others 2005), this study provides evidence of the potential for host–vector interaction with UK equines. *Cs. annulata* has recently been demonstrated to be vector competent for WNV (M. S. C. Blagrove, personal communication), and as the species bites both birds and mammals including horses (Schönenberger and others 2016), it therefore has potential to transmit arboviruses from avian reservoirs and hence serve as a 'bridge vector'. Combined with its ability to breed in a variety of water sources and presence on most sites sampled, this makes it an important species for further study.

The authors' results suggest that mosquito species presence is determined mainly by local mosquito breeding habitat rather than equine host availability or management factors. However, biting of horses may be affected by practices such as use of repellents, rugs and masks, building design, and duration and timing of grazing.

Mosquito magnets are a commonly used trap in Europe for surveillance. They catch almost all mammalophagic species of mosquito, and catch more species than other systems and in greater numbers. BG sentinel and Centers for Disease Control and Prevention traps were not used in this study as they were considered less suitable due to the risk of unpredictable precipitation damaging samples, and because for wide-scale trapping in the UK, it may prove more practicable to use propane vendor's delivery services than to transport large amounts of dry ice or carbon dioxide. Red-box traps were used in the current study to attempt to trap blood-fed mosquitoes; however, no mosquitoes were captured. Similar but larger red-box traps have been successful in capturing *An. maculipennis s.l.*, *Cs. annulata* and *Culex* species in England (Brugman 2016). At similar future levels of mosquito abundance and without the presence of invasive species, surveillance on equine premises in the UK should be based around the use of mosquito magnets and larval sampling.

Mosquito populations often have a patchy distribution (Medlock and others 2005, Snow and Medlock 2008, Golding 2013) and many are considered uncommon or rare. Simple random sampling of equine premises may have resulted in very low catches. It is also almost impossible to prove species absence, so using random sampling risked obtaining poor quality data. Stratified sampling is an alternative method, commonly used by ecologists studying rare species (Thompson 2012). Using the data obtained under this sampling regimen, it is not possible to estimate the risk of equine–mosquito interaction across the UK, but more accurate assessment of risk at individual sites based on local habitat is achievable.

There are a number of introduction pathways that could conceivably be involved in importation of arboviral disease to the UK. Perhaps the most often discussed is introduction of WNV by migratory birds, but trade and transport of exotic birds and pets, and inadvertent vector transportation are also relevant risks. There is some recent evidence that human populations may continue epidemic transmission of VEEV in urban environments (Bowen and Calisher 1976, Watts and others 1998, Morrison and others 2008). Therefore in the event of an outbreak in the Americas, human movements as well as horse movements may constitute a risk (Adams and others 2012). Livestock transport, human transport and possibly mosquito eggs may present risk of RRV introduction (Harley and others 2001). Due to the complexity of the transmission cycles, virus introduction may not result in autochthonous (in-country) transmission.

In conclusion, the current study has highlighted a number of mosquito species that should be investigated with regard to vector competence and effectiveness of protection measures for equines. The authors' work has shown that horses in the UK are at risk of attack from a wide variety of mosquito species, several of which are known to be vectors of equine arboviruses in affected countries.

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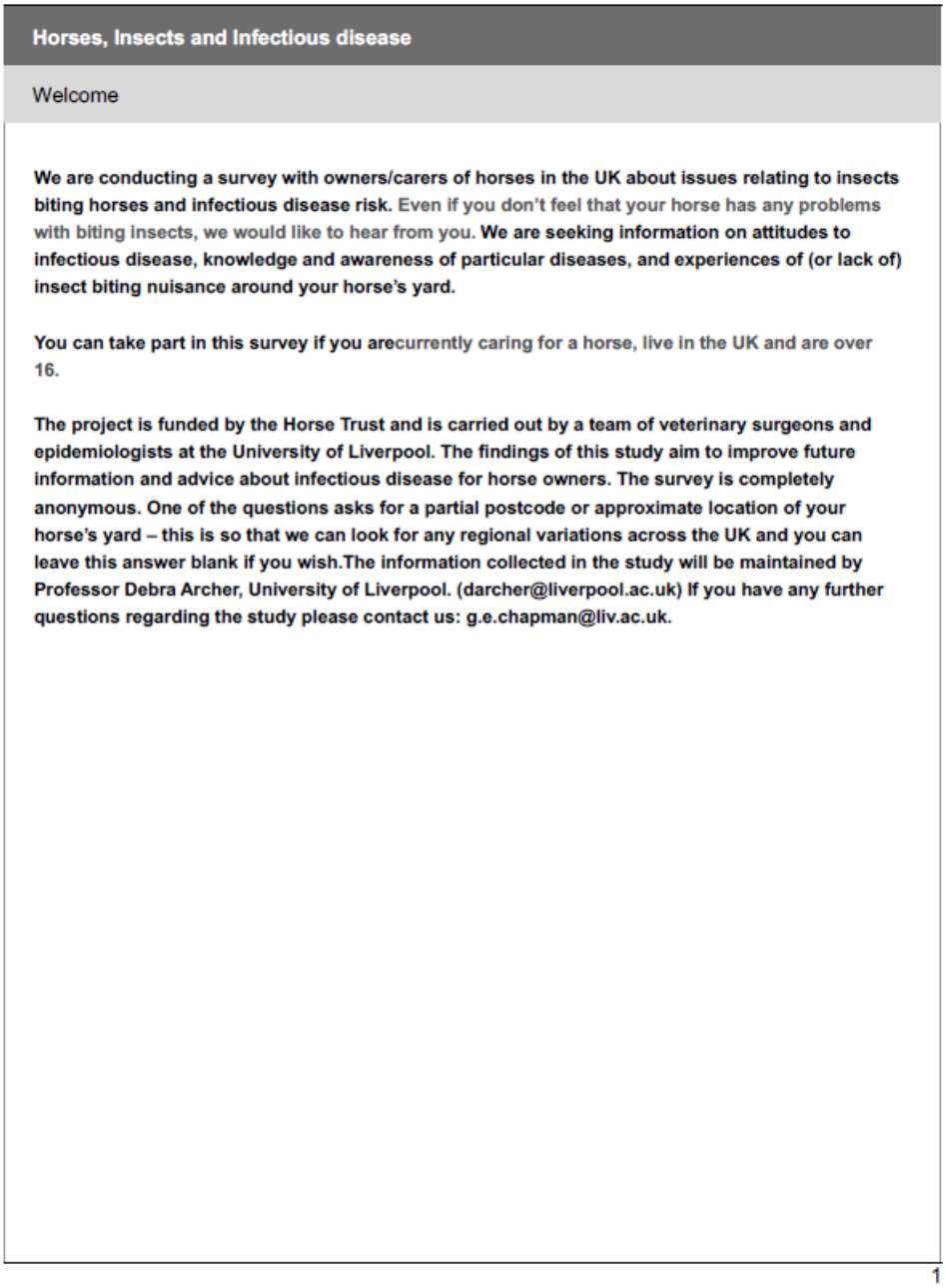
Appendix C (Questionnaire – Chapter 2)



Horses, Insects and Infectious disease

Welcome

We are conducting a survey with owners/carers of horses in the UK about issues relating to insects biting horses and infectious disease risk. Even if you don't feel that your horse has any problems with biting insects, we would like to hear from you. **We are seeking information on attitudes to infectious disease, knowledge and awareness of particular diseases, and experiences of (or lack of) insect biting nuisance around your horse's yard.**

You can take part in this survey if you arecurrently caring for a horse, live in the UK and are over 16.

The project is funded by the Horse Trust and is carried out by a team of veterinary surgeons and epidemiologists at the University of Liverpool. The findings of this study aim to improve future information and advice about infectious disease for horse owners. The survey is completely anonymous. One of the questions asks for a partial postcode or approximate location of your horse's yard – this is so that we can look for any regional variations across the UK and you can leave this answer blank if you wish.The information collected in the study will be maintained by Professor Debra Archer, University of Liverpool. (darcher@liverpool.ac.uk) If you have any further questions regarding the study please contact us: g.e.chapman@liv.ac.uk.

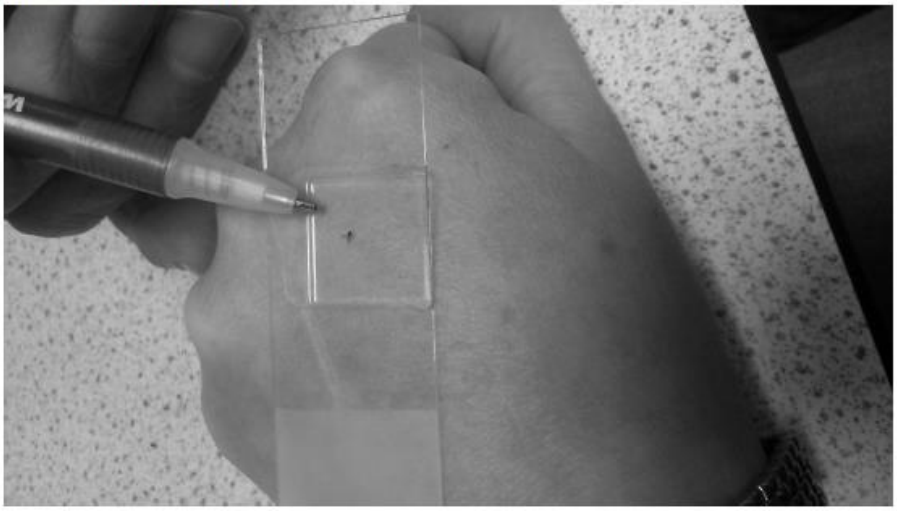
1

Horses, Insects and Infectious disease

Insect 1 (Image attribution: CSIRO [CC BY 3.0 (http://creativecommons.org/licenses/by/3.0)], via Wikimedia Commons)

2

Insect 1 - Showing actual size



1. What would you call the insect above?

- ◯ Mosquito
- ◯ Midge
- ◯ Stable Fly
- ◯ Horse Fly
- ◯ Gnat
- ◯ I have never seen this before
- ◯ I do not know
- ◯ Other (please specify)

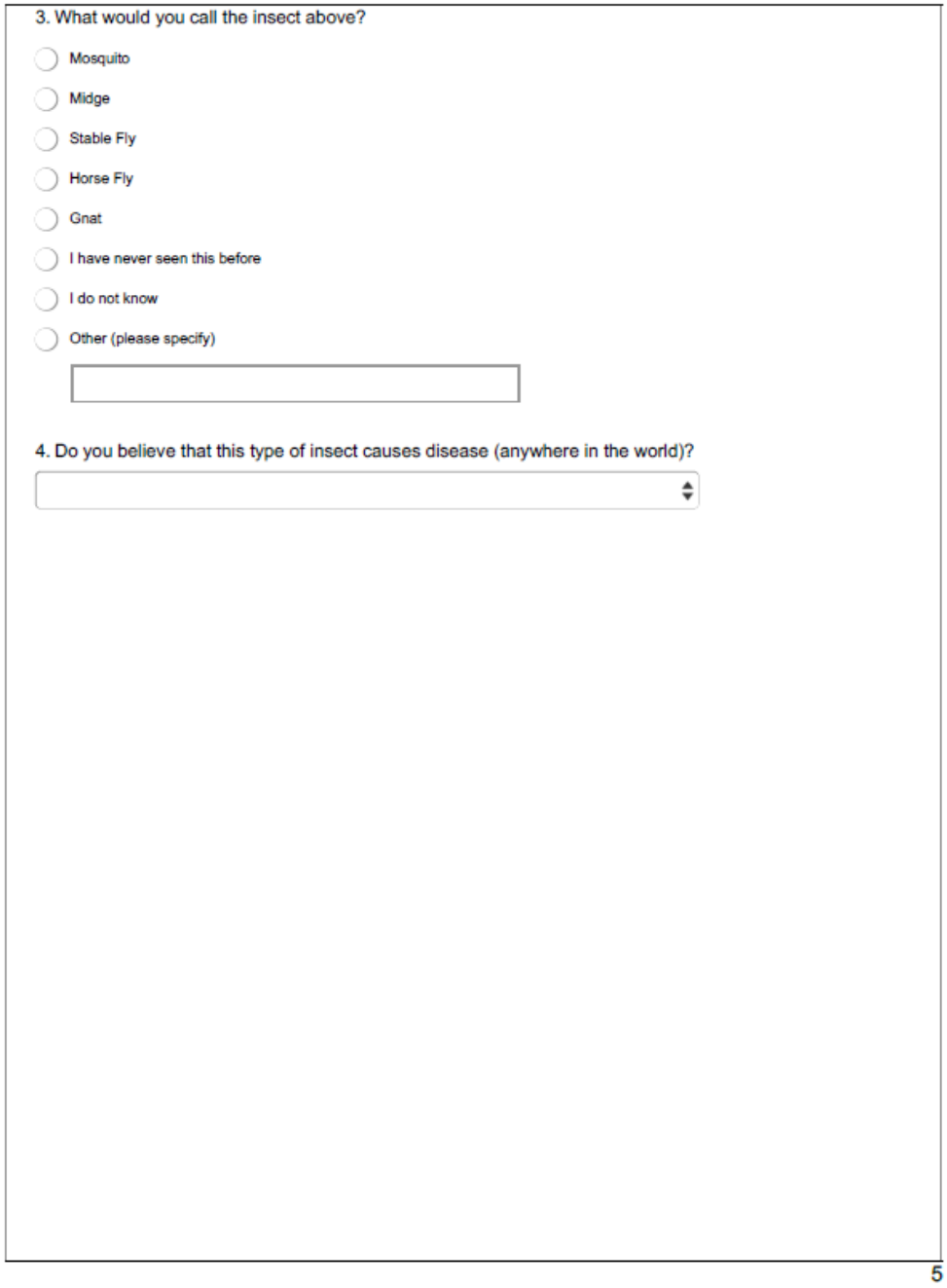
* 2. Do you believe that this type of insect causes disease (anywhere in the world)?

3

Horses, Insects and Infectious disease

Insect 2 (Author: Abeer Jabbar. Available under public license https://www.flickr.com/photos/abeerjabbar/3296527723, http://creativecommons.org/licenses/by-sa/4.0)

4



3. What would you call the insect above?

- Mosquito
- Midge
- Stable Fly
- Horse Fly
- Gnat
- I have never seen this before
- I do not know
- Other (please specify)

4. Do you believe that this type of insect causes disease (anywhere in the world)?

5

Horses, Insects and Infectious disease

Insect 3

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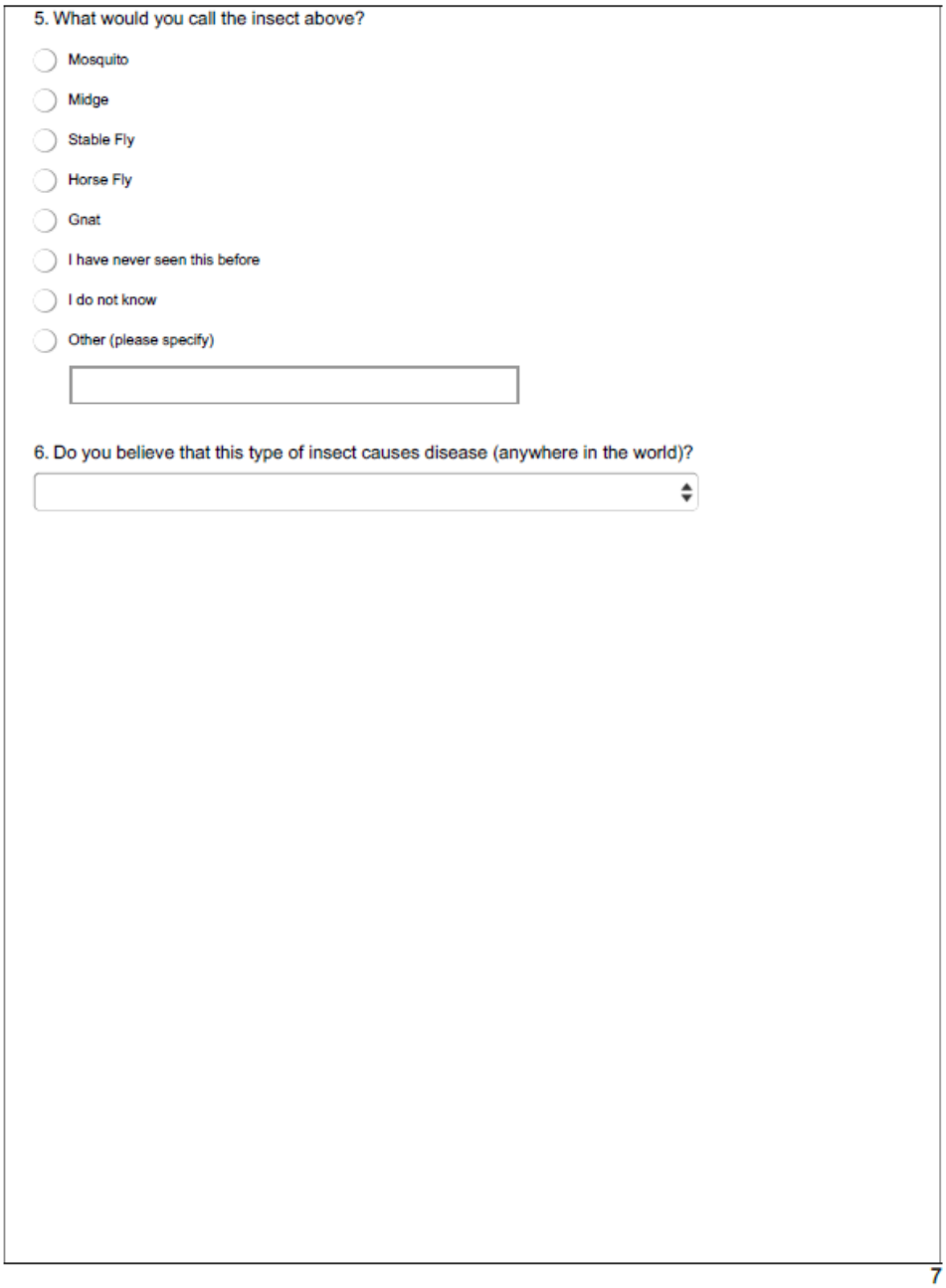
6



5. What would you call the insect above?

- Mosquito
- Midge
- Stable Fly
- Horse Fly
- Gnat
- I have never seen this before
- I do not know
- Other (please specify)

6. Do you believe that this type of insect causes disease (anywhere in the world)?

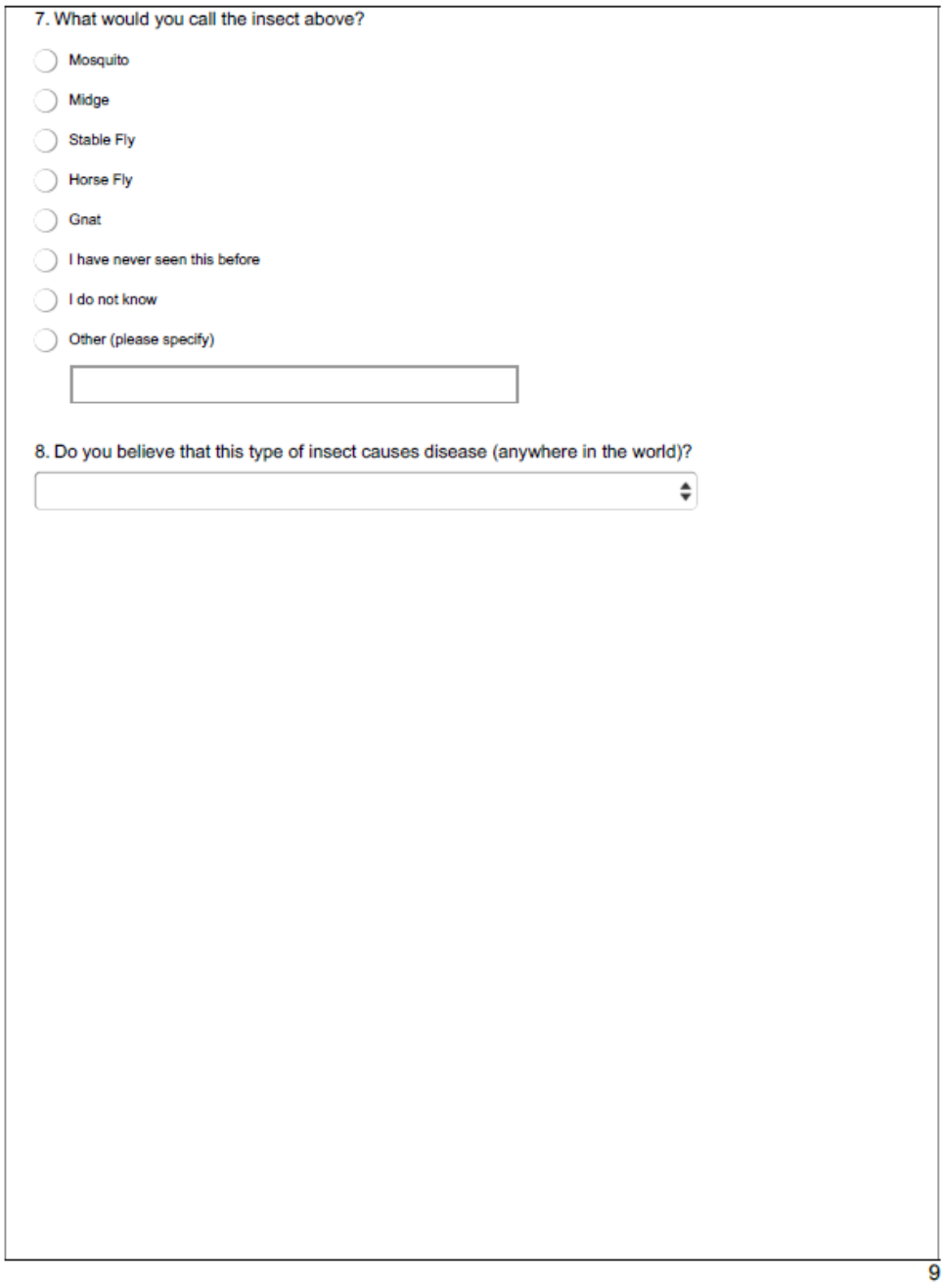
7

Horses, Insects and Infectious disease

Insect 4

(Image attribution: By Sandy Rae from Scotland, UK [CC BY 2.0 (http://creativecommons.org/licenses/by/2.0)], via Wikimedia Commons)

8



7. What would you call the insect above?

- Mosquito
- Midge
- Stable Fly
- Horse Fly
- Gnat
- I have never seen this before
- I do not know
- Other (please specify)

8. Do you believe that this type of insect causes disease (anywhere in the world)?

9

Horses, Insects and Infectious disease

9. Are you aware of biting insects on the premises where you keep your horse? (Check all that apply)

- [ ] Mosquitoes
- [ ] Midges
- [ ] Horse flies
- [ ] Stable flies
- [ ] Yes, but I'm not sure which
- [ ] Other biting insects (please specify)

10. What makes you aware of the presence of these insects on your yard (please check all that apply)

	MOSQUITOES	MIDGES
I am not aware of them	☐	☐
I see them on horses at pasture	☐	☐
I see them on horses in the stable	☐	☐
I see them biting horses	☐	☐
Horses are irritated by them	☐	☐
I get bitten	☐	☐
I see swarms of insects	☐	☐
Other	☐	☐

11. Do you feel that MOSQUITOES cause a problem on your yard, during the warmer months to any of the horses or people?

No	A minor problem	A moderate problem	A major problem
○	○	○	○

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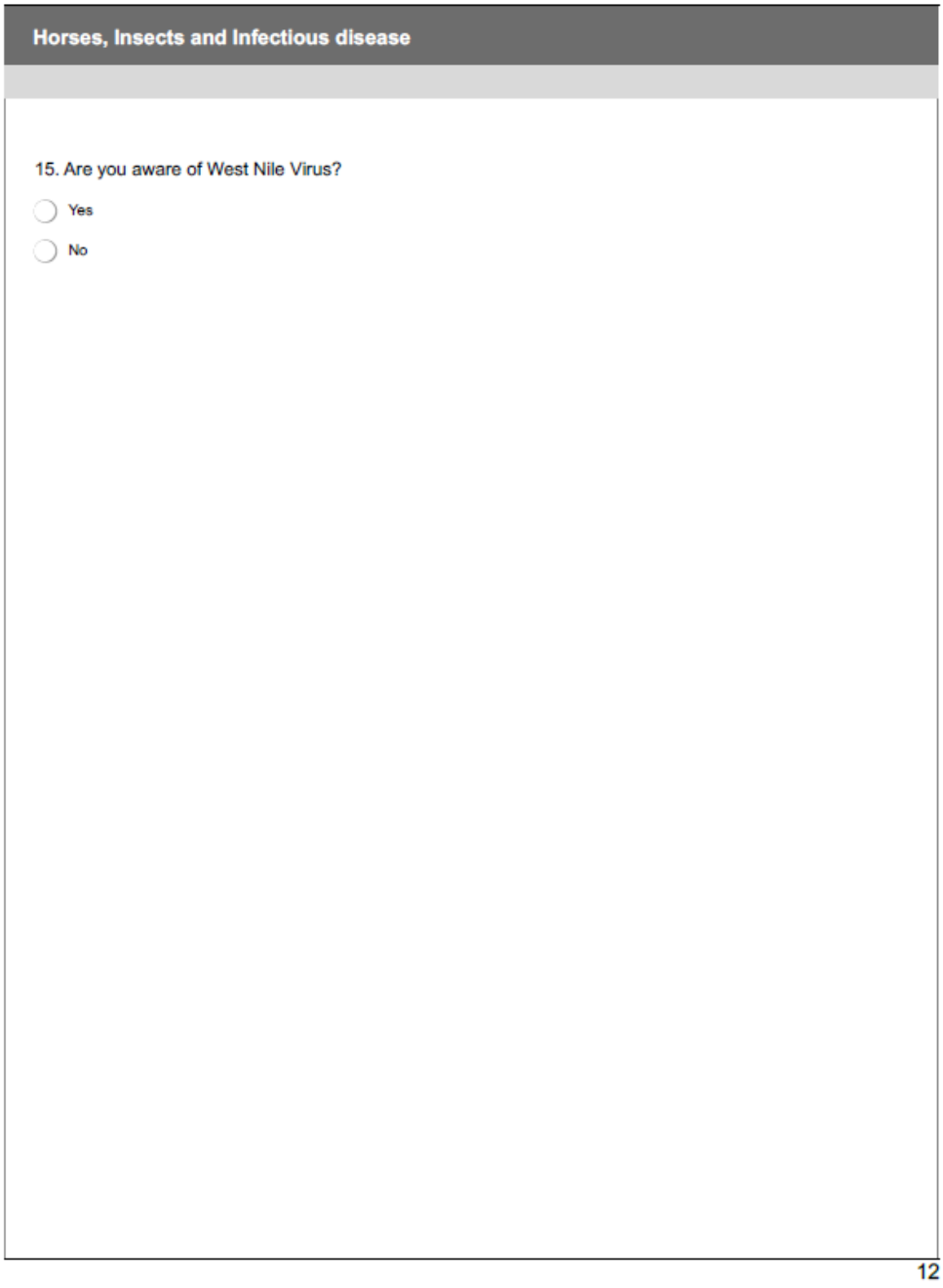
12. Do you feel that MIDGES cause a problem on your yard, during the warmer months to any of the horses or people?

No	A minor problem	A moderate problem	A major problem
○	○	○	○

13. Are there any diseases that you are aware of that MIDGES can pass to horses (worldwide)?

14. Are there any diseases that you are aware of that MOSQUITOES can pass to horses (worldwide)?

11



Horses, Insects and Infectious disease

15. Are you aware of West Nile Virus?

- ◯ Yes
- ◯ No

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Horses, Insects and Infectious disease

16. Do you know if West Nile Virus can affect horses?

- ◯ Yes it can
- ◯ No it cannot
- ◯ I do not know

17. Have you heard of African Horse Sickness?

- ◯ Yes
- ◯ No

18. Are you aware of any other insect-borne diseases of horses (which occur anywhere in the world)?

- ◯ No
- ◯ Yes (please specify)

13

Horses, Insects and Infectious disease

19. Please check the box you believe is correct for each statement. Currently there are around 900,000 horses in the UK. If there were an outbreak of African Horse Sickness in the UK:

	True	False	I don't know
The disease could spread rapidly through the UK	○	○	○
Many horses could become ill	○	○	○
Horses could die from the disease	○	○	○
Lots of horses (more than 1000) could die from the disease	○	○	○
The government would ban movement of horses in affected areas	○	○	○
A vaccination campaign would be necessary to prevent further spread	○	○	○
Vaccination could be done immediately to protect horses	○	○	○

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20. Please check the box you believe is correct for each statement.
If there were an outbreak of West Nile virus in the UK:

	True	False	I don't know
The disease could spread rapidly through the UK	○	○	○
Many horses could become ill	○	○	○
Horses could die from the disease	○	○	○
Lots of horses (more than 1000) could die from the disease	○	○	○
The government would ban movement of horses in affected areas	○	○	○
A vaccination campaign would be necessary to prevent further spread	○	○	○
Vaccination could be done immediately to protect horses	○	○	○

21. Please describe what signs you believe a horse might show if it was infected with West Nile Virus. If you are not aware of any at all, leave blank.

22. Please describe what signs you believe a horse might show if it was infected with African Horse Sickness. If you are not aware of any at all, leave blank.

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Horses, Insects and Infectious disease

23. There is a vaccine for West Nile virus. Assuming it was priced around £30-£35 per vaccine, similar to a combined flu and tetanus vaccine - (2 vaccinations in the first year then a booster each year) and you would pay your usual veterinary call out fee, would you have your horse vaccinated if an outbreak of disease occurred in the UK?

- ◯ Yes
- ◯ No
- ◯ Maybe

24. What might stop you from having your horse vaccinated?

25. Do you routinely have your horse(s) vaccinated for:

- ☐ Influenza?
- ☐ Tetanus?
- ☐ Other (please specify)

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Horses, Insects and Infectious disease

26. Please indicate if any of these applies to your horse, or toALL of your horses, if you have more than one:

- ☐ My horse is / horses are not ridden / driven
- ☐ My horse is / horses are at pasture permanently and rarely use a stable (please check this even if they use a field shelter)
- ☐ My horse is / horses are never or rarely kept in the stable in the summer months (e.g. just to tack up, wait for farrier etc)

These are not relevant as my horse's management do not fit these criteria. (Please describe your horse's management).

27. Do you use any of these methods to reduce insect bites to your horse? (Check all that apply)

	In the stable	At pasture	Only when ridden / driven
Apply repellent when the horse is:	☐	☐	☐
Use fly rugs / exercise sheet when the horse is:	☐	☐	☐
Use fly masks or fringes when the horse is:	☐	☐	☐

28. Do you use any of these methods to reduce insect bites to your horse? (Check all that apply)

- ☐ Barriers such as fly screens to prevent insects entering the stable
- ☐ Wash in insecticides / repellents such as Deosect, Coopers Fly Repellent Plus, or similar
- ☐ None of the these
- ☐ Spray on Fly repellent - Please specify which product(s) you use.

29. If you use any other methods to reduce insect bites, or would like to comment on how you combine methods for increased protection, please describe here:

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30. Are there any bite reduction methods that you feel are useful against one insect but not another. If so please describe:

31. Who would you currently ask for advice on insect control in relation to your horse? (Check all that apply)

- [ ] Veterinary surgeon
- [ ] Yard Owner
- [ ] Tack shop staff
- [ ] Others on your yard
- [ ] Internet sources
- [ ] Other (please specify)

32. If you answered that you use the internet in the previous question, please describe which sources you would use to find out about protecting horses insect bites

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Horses, Insects and Infectious disease

33. If there were an outbreak of insect-borne disease in the UK, would you seek information about disease control, or protection from insects from a different source?

- ○ No
- ○ Yes - Please give your thoughts on this

34. Do you have any other comments about insect-borne disease?

35. If you are happy to do so, please supply the first four digits of the postcode, or nearest town and the county of your yard.

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Horses, Insects and Infectious disease

Thank you for completing this survey

Thank you for completing our survey. If you would like further information about mosquito-borne viruses or the project it is available here.
Please remember that no horse has ever caught any of these diseases in the UK, and none of the diseases mentioned in this survey or considered in this project is currently present. Also, there are vaccinations available for the majority of these diseases in countries affected. If you have any further questions or concerns regarding these diseases you are welcome to contact us: g.e.chapman@liv.ac.uk.

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